Remarks

I. Status of the Claims

Reconsideration of this Application is respectfully requested. Upon entry of the foregoing amendment, claims 1-4, 6-9 and 14-33 are pending in this application, with claims 1, 21 and 32 being the independent claims. Claims 5 and 10-13 are cancelled without prejudice or disclaimer of the subject matter therein. Claim 19 is withdrawn. Claims 1-2, 4, 6-9, 14-25 and 27 are amended and claims 28-33 are new. Support for the amendment and addition of claims may be found in the specification, for example, at paragraphs 0022, 0031, and 0033 and in the original claims. Accordingly, the amendments introduce no new matter.

Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and further request that they be withdrawn.

II. Restriction and Election of Species

The Examiner has partially withdrawn the Restriction Requirement of December 30, 2005 and rejoined and examined together the claims of Groups I-III. (Office Action, p. 2, ¶2.) The Examiner has maintained the Restriction Requirement as to Group IV and, accordingly, has withdrawn from consideration claim 19.

The Examiner has examined the pending claims to the extent that they read upon the elected species of HIV. Upon allowance of generic or linking claims, Applicants are entitled to allowance of a reasonable number of species in addition to the elected species. *See* 37 C.F.R. § 1.146.

III. The Specification

The Examiner indicated that Applicants are required to update the status of all parent priority applications. (Office Action, pp. 2-3, ¶4.)

The captioned application claims priority to a provisional patent application. Provisional applications by law are abandoned one year after the filing date. Applicants respectfully submit that there is no requirement to provide status information on provisional patent applications.

At pages 2-3, ¶4, of the Office Action the Examiner also stated that "[t]he status of all citations of US filed applications in the specification should also be updated where appropriate." Applicants respectfully traverse this requirement. The Examiner has not cited to any authority that requires Applicants to update the status of all cited, U.S.-filed applications. Accordingly, Applicants assert that this requirement is in error and should be withdrawn.

IV. The Information Disclosure Statement

Applicants respectfully request that the Examiner consider and intial IDS documents AR13, AS13, and AT13, listed on page 13 of the Information Disclosure Statement filed on May 7, 2004, so that the record is clear in regards to these documents.

V. Rejection under 35 U.S.C. § 112, first paragraph: Written Description

The Examiner has rejected claims 1-18 and 20-27 as allegedly not complying with the written description requirement under 35 U.S.C. § 112, first paragraph. (Office Action, pp. 3-6, ¶¶6-14). The Examiner asserts that Applicants did not possess the entire scope of the claims, in particular, for the recitation of "cell, virion, pseudovirion, membrane vesicle, lipid bilayer, or liposome" and for any confirmation specific antibody against any virus.[1]

Applicants have amended the claims to recite a "membrane bound" viral envelope protein or "membrane bound" fragment thereof. The claims no longer recite "a cell, virion, pseudovirion, membrane vesicle, lipid bilayer, or liposome."

Applicants respectfully traverse the rejection as applied to the original or amended claims. As explained below, the Examiner's basis for the rejection is unsupported by the evidence and is contrary to the current state of the law concerning the written description requirement.

(a) The Specification Describes A Number of Membrane Types, Viral Envelope Proteins, and Confirmation-Specific Antibodies

Concerning the term "membrane bound," as noted in the specification, a viral envelope protein can be associated with a lipid bilayer in a number of different ways as long as the environment allows the formation of conformational changes in the envelope protein that are associated with membrane fusion. (Specification, ¶¶ 0023 and 0055.) Different kinds of membranes were known in the art that could be used with viral envelope proteins in, for example, membrane fusion assays. For example, as noted in the specification:

> Cells expressing the envelope glycoprotein or fragment thereof are cells infected with a recombinant vaccinia virus expressing the HIV-1 envelope protein or fragment thereof. In another embodiment, the cells expressing the envelope

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[1] Applicants note that although the Examiner has focused on conformation-specific antibodies, the claims also encompass the use of antibodies that are not specific for induced conformations, e.g., antibodies that bind "epitopes present only prior to induction of entry-related conformational changes." (Specification, ¶ 0045.)

> glycoprotein or fragment thereof are cells transformed with a vector expressing the HIV-1 envelope protein or fragment thereof. In another embodiment, the cells expressing the envelope glycoprotein or fragment thereof are infected with a replication defective viral particle or pseudovirion bearing at least one envelope protein or fragment thereof from at least one laboratory-adapted or primary virus infected cells.
>
> Useful lipid bilayer systems include cells, virions, pseudovirions or other appropriate membrane vesicles or liposomes expressing or bearing either a viral envelope protein or glycoprotein or fragments thereof. The envelope viral protein or glycoprotein will typically have one or more membrane-associating domains and one or more transmembrane domains. Examples of useful lipid bilayer systems in the present invention include: cells transfected such that they surface express membrane associated envelope protein or glycoprotein, cells infected with replication defective viral particles and surface expressed membrane associated envelope protein or glycoprotein, inactivated virus particles, and pseudovirions.

(Specification, pp. 16-17, ¶¶ 0056-0057.)

The specification also sets forth specific exemplary membranes useful in the claimed method:

- Non-infectious HIV-1 particles such as 8E5/LAV virus (Folks, T.M., et al., J. Exp. Med. 164:280-290 (1986); Lightfoote, M.M., et al., J. Virol. 60:771-775 (1986); Gendelman, H.E., et al., Virology 160:323-329 (1987)) (Specification, p. 18, ¶ 0065);

- Pseudovirions bearing the envelope glycoprotein or fragment thereof from at least one laboratory-adapted or primary HIV-1 isolate or virus infected cell (Haddrick, M., et al., J. Virol. Methods 61:89-93 (1996); Yamshchikov, G.V., et al., Virology 21:50-58 (1995)) (Specification, p. 18, ¶ 0065);

- The 8E5/LAV cell line, which produces an intact virion expressing functional envelope in a non-replicating system (Specification, p. 18, ¶ 0066);

- Cells infected with a recombinant vaccinia virus expressing the HIV-1 envelope protein or fragment thereof (Earl, P.L., et al., J. Virol. 65:31-41 (1991); Rencher, S.D., et al., Vaccine 5:265-272 (1997); Katz, E. and Moss, B., AIDS

Res. Hum. Retroviruses 13:1497-1500 (1997)) (Specification, p. 18-19, ¶ 0067); and

- H9 cells expressing HIV-1 envelope (Specification, p. 45, ¶ 0136).

Concerning viral envelope proteins, the specification describes a number of viruses whose envelope proteins may be used in the claimed method. The specification states:

> The method of the present invention can be applied to viruses where a transmembrane protein or glycoprotein forms structures, conformations, and complexes that are involved with virus entry, including but not limited to, HIV-1, HIV-2, HTLV-I, HTLV-II, respiratory syncytial virus (RSV), parainfluenza virus type 3 (HPIV-3), human influenza viruses, measles virus, hepatitis B virus (HBV) or hepatitis C virus (HCV) or other enveloped viruses.

(Specification, p. 17, ¶ 0058.) Additional viruses are described on page 9, paragraph 0026 (retroviruses), and page 19, paragraph 0071 (Newcastle disease virus). Since the envelope protein sequences for these viruses are known, it was not necessary for Applicants to include the sequences in the specification. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) ("a patent need not teach, and preferably omits, what is well known in the art."). (*See*, also, section V(c), below.)

In addition, concerning antibodies against conformational intermediates of viral envelope proteins, the specification provides a number of such antibodies, and guidance for producing additional ones. For example, as is noted in the specification, conformation-specific antibodies against HIV had been produced prior to the effective filing date. These antibodies include:

- T26 (Earl et al, J Virol 1997 Apr;71(4):2674-84) (Specification, p. 15, ¶ 0051);

- NC-1 (Jiang et al, J Virol 1998 Dec;72(12):10213-7) (Specification, p. 15, ¶ 0051);
- 17b (Sullivan et al, J Virol 1998 Jun;72(6):4694-703) (Specification, p. 15, ¶ 0052);
- 48d (Thali et al, J Virol 1993 Jul;67(7):3978-88) (Specification, p. 15, ¶ 0052),
- 8F101 (DeVico et al, Virology 1995 Aug 20;211(2):583-8) (Specification, p. 15, ¶ 52); and
- A32 (Wyatt et al, J Virol 1995 Sep;69(9):5723-33) (Specification, p. 15, ¶ 52).

The specification also provides guidance for producing additional conformation-specific antibodies. As taught in the specification, conformation-specific antibodies can be produced by immunizing an animal with a mixture of peptides or recombinant proteins that mimic the 6-helix bundle structure of the envelope protein. (Specification, ¶ 0051.) More than 20 pages of disclosure teach, in detail, how to produce antibodies against these conformational intermediates. (Specification, pp. 20-45) In particular, the specification discloses a number of peptide sequences that are useful for generating antibodies against entry-related structures (SEQ ID NOs:1-77). The peptides disclosed include some from RSV, HPIV3 and measles virus (SEQ ID NOs:75-77). The specification also cites, and incorporates by reference, five U.S. patents and a PCT publication that disclose useful peptides. (Specification, ¶ 0103.) The disclosed methods would allow a person of ordinary skill in the art to readily identify a multitude of antibodies that can be used in the practice of the claimed methods.

The Examiner has not explained why the aforementioned exemplary membranes, viral envelope proteins, antibodies and methods of producing additional antibodies are deemed insufficient to adequately describe the genus of reagents recited by the present claims.

(b) The Level of Skill In the Art Relating to the Membranes, Viral Envelope Proteins and Conformational Antibodies Was Extremely High

When assessing the adequacy of written description provided for a particular claimed invention, it is necessary to consider the level of skill in the art. As articulated recently by the Federal Circuit:

> The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

Capon v. Eshhar, 418 F.3d at 1357, 76 U.S.P.Q.2d at 1084. Here, the level of skill and knowledge in the art relating to the production and use of membranes, viral envelope proteins, and conformational antibodies was extremely high at the time of the effective filing date of the present application.

For example, concerning membranes, a great deal was known of the structure and properties of membranes such as lipid bilayers and vesicles. *See*, e.g., Small, D.M., "Lipids, Structure and Biochemistry of," in *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, Meyers, R.A., ed., Wiley-VCH, New York, pp. 503-9 (1995) (describing the structure and properties of lipid bilayers, vesicles and cell membranes, and the lipids of which these structures are composed) (copy attached as Exhibit 1). In addition, pure lipid bilayers had been studied extensively for the purpose of understanding membrane fusion. *See*, e.g., "Membrane Fusion, Molecular Mechanisms of," in *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, Meyers, R.A., ed., Wiley-VCH, New York, pp. 537-41 (1995) (copy attached as Exhibit 2).

In addition, the sequence of numerous viral envelope proteins was also known. For example, the sequence of a human hepatitis virus was first published in 1979 (Charnay *et al., Nucl. Acids Res. 7*:335-46 (1979); Valenzuela *et al., Nature 280*:815-19 (1979); Galibert *et al., Nature 281*:646-50 (1979); and Pasek *et al., Nature 282*:575-79 (1979)), the sequence of a Rous sarcoma virus was first published in 1983 (Hunter *et al., J. Virol. 46*:920-36 (1983)), an HTLVII envelope protein sequence was published in 1984 (Sodrooski *et al., Science 225*:421-4 (1984)), and various envelope sequences of HIV have been published, beginning in the mid 1980s (e.g., Modrow *et al., J. Virol. 61*:570-8 (1987) (citing five articles disclosing envelope genes from seven HIV isolates)).[2] Numerous other viral envelope sequences were published in the 20+ years spanning 1979 to the effective filing date of the present application.

Moreover, the level of skill concerning conformational intermediates and antibodies thereto was also extremely high. In particular, the conformational changes leading to a fusion-active state have been well described in the literature. Eckert and Kim, "Mechanisms of Viral Membrane Fusion and Its Inhibition," *Annu. Rev. Biochem. 70*:777-810 (2001), pp. 780-793 (copy attached as Exhibit 4). Skilled artisans have created a common model of viral fusion based on the detailed knowledge of the influenza HA protein and the HIV envelope protein. *Id.*, abstract; p. 778, last paragraph; p. 780, first full paragraph; p. 786, last paragraph. X-ray crystal studies on the fusion protein cores of orthomyxoviruses, retroviruses, paramyxoviruses, and filoviruses showed that these virus families have a fusion-active state very similar to that of HIV and influenza. *Id.*, p. 793 (section titled "Other Membrane Fusion Proteins"). In addition, an improved computational method had been developed that allows "more accurate predictions for many viruses in the retrovirus, paramyxovirus, and filovirus families." *Id.* Structures that were predicted using this method were subsequently confirmed for a number of viruses. *Id.*

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[2] A copy of the abstract of each article cited in this paragraph is attached as Exhibit 3.

Given the ability to accurately predict which residues participate in the conformational intermediate structures for any given viral envelope protein, one of ordinary skill could have readily produced antibodies specific for those structures.

The Examiner has not presented any evidence or argument to suggest that the level of skill and knowledge in the relevant art was not high. In fact, it appears that the Examiner has not considered the level of skill and knowledge in the art at all in the context of analyzing the written description requirement. For this reason alone -- in view of the Federal Circuit's admonition in *Capon* that the state of the art must be considered in assessing adequacy of written description -- the present rejection cannot stand.

(c) The Claim Terms Alone Readily Convey Distinguishing Information Concerning the Reagents

Applicants submit that the Examiner's analysis of the written description requirement is flawed because it focuses improperly on elements of the claims that are not the point of novelty of the invention. Several recent opinions of the Federal Circuit confirm that, for generic elements of a claim that are well known in the art and are not themselves the point of novelty of a claimed invention, the written description requirement may be satisfied with respect to those elements by their recitation alone.

For example, in *Amgen*, the Federal Circuit held that a patent specification that disclosed only two species of vertebrate or mammalian cells nonetheless provided adequate written description support for method claims that involved the use of vertebrate or mammalian cells, generally. *See Amgen Inc. v. Hoechst Marion Rousel Inc.*, 314 F.3d at 1332, 65 U.S.P.Q.2d at 1398. According to the court:

> the claim terms at issue here are not new or unknown biological materials that ordinary skilled artisans would easily miscomprehend. Instead, the claims of Amgen's patents refer to types of cells that can be used to produce recombinant human EPO...*the words "vertebrate" and "mammalian" readily "convey[] distinguishing information concerning [their] identity" such that one of ordinary skill in the art could "visualize or recognize the identity of the members of the genus."* Indeed, the district court's reasoned conclusion that the specification's description of producing the claimed EPO in two species of vertebrate or mammalian cells adequately supports claims covering EPO made using the genus of vertebrate or mammalian cells, renders *Eli Lilly* listless in this case.

Id. (internal citations omitted, emphasis added). The court's decision was based on two principle factors:

- That the claim terms at issue ("vertebrate" and "mammalian") did *not* refer to new or unknown biological materials that ordinary skilled artisans would easily miscomprehend; and
- That the words "vertebrate" and "mammalian," as used in the claims, readily conveyed distinguishing information concerning their identity such that one of ordinary skill in the art could visualize or recognize the identity of members of the genus.

When the reasoning of *Amgen* is applied in the context of the present claims, it is clear that the written description requirement is more than adequately satisfied for claims reciting any membrane bound viral envelope protein or fragments thereof, as well as confirmation-specific antibodies against the viral envelope protein.

First, the term "membrane bound" viral envelope protein or fragment thereof, like the terms "vertebrate" and "mammalian," does not refer to new or unknown biological materials that ordinary skilled artisans would easily miscomprehend. As noted above in section V(b), membranes, including lipid bilayers and vesicles, had been studied extensively and their structures were known. Likewise, viral envelope proteins, conformational intermediates thereof, and antibodies thereto were also know in the literature. *See* sections V(a) and (b), above. Thus, the reagents recited in the present claims were not new or unknown biological materials (although their usefulness in identifying inducers of conformational intermediates was not appreciated prior to the present invention).

Second, the claim terms themselves readily convey distinguishing information concerning the identity of the reagents so that persons of ordinary skill in the art could recognize the identities of members of the genus. Persons of ordinary skill in the art would readily understand from the claim terms alone that the reagents used in the practice of the claimed methods are (a) membrane bound viral envelope proteins or membrane bound fragments thereof, and (b) envelope-specific antibodies (e.g., conformation-specific antibodies, antibodies specific for envelope proteins prior to conformation induction, or antibodies specific for HIV-1 gp120). Thus, a skilled person would be able to readily distinguish the reagents used in the practice of the claimed methods from proteins and antibodies that fall outside the scope of the claim language.

In summary, under *Amgen*, the expressions "membrane bound envelope protein or membrane bound fragment thereof," and determining "binding of an antibody . . . to said membrane bound viral envelope or fragment thereof," by themselves, convey sufficient identifying information regarding the reagents so that a person of ordinary skill in the art could visualize or recognize members of the recited genus.

The general approach to the written description inquiry set forth in *Amgen* has recently been confirmed by the Federal Circuit. For instance, in *Capon*, the Federal Circuit held that, in the context of claims to chimeric genes comprising *known* genetic elements, it was unnecessary for the applicants/patentees to provide a structural description (*i.e.*, a recitation of the nucleotide sequence) of the claimed chimeric genes. *See id.*, 418 F.3d at 1358, 76 U.S.P.Q.2d at 1084-85. According to the court, "[w]hen the prior art includes the nucleotide information [of the component DNAs], precedent does not set a *per se* rule that the information must be determined afresh." *Id.* This rationale was further endorsed in the recent case of *Falkner v. Inglis*, 448 F.3d 1357, 2006 U.S. App. LEXIS 13127 (Fed. Cir. May 26, 2006) ("it is the binding precedent of this court that *Eli Lilly* does *not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art.") Applied to the circumstances of the present application, *Capon* and *Falkner* strongly support Applicants' position that the genus of reagents recited in the claims is more than adequately described by virtue of the fact that the molecules identified in the present application as viral envelope proteins and antibodies thereto were known in the prior art.

(d) Example 18 of the USPTO's Written Description Guidelines Indicates that The Genus of Reagents Defined in the Claims is Adequately Described

Example 18 of the USPTO's "Synopsis of Application of Written Description Guidelines" (available at http://www.uspto.gov/web/menu/written.pdf, copy attached as Exhibit 5) provides further support for Applicants' position. This Example illustrates an analysis of the written description provided for a process claim where the novelty is in the method steps. The claim at issue in this Example is as follows:

> A method of producing a protein of interest comprising;
> obtaining *Neurospora crassa* mitochondria,
> transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,
> expressing said protein in said mitochondria, and
> recovering said protein of interest.

The specification in Example 18 shows actual reduction to practice of a single embodiment: the expression of β-galactosidase. The claimed process, however, involves the use of *any* nucleic acid that encodes *any* protein of interest, a virtually unlimited genus. Nonetheless, the Example concludes that the claimed invention is adequately described. According to the analysis provided in this Example:

> The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.
> The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and

> knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Significantly, in assessing the written description of this hypothetical claim, the USPTO's Example does not even question whether the specification provides adequate description of the entire genus of nucleic acid encoding a protein of interest because the nucleic acid is *not itself being claimed.* Thus, the emphasis in this analysis is on whether the *process* is adequately described. Structural information regarding the individual *elements* used in carrying out the process is clearly *not* required to satisfy the requirements of § 112, first paragraph under these circumstances.

Analogously, the reagents recited in the present claims -- membrane bound viral envelope proteins and antibodies against conformational intermediates -- are not themselves being claimed; they are simply elements used in the practice of the claimed methods. Thus, the written description analysis should focus on whether or not the *methods* are adequately described, not whether these reagents are adequately described (although they certainly are adequately described by, *inter alia*, the numerous exemplary substrates described in the specification in view of the advanced state of the art. *See* sections V(a) and (b), above).

As explained above, one of the principles taught in Example 18 is that the written description inquiry should focus on the novel aspects of the invention (*e.g.*, the process steps of the claimed method) rather than on elements of the claims that do not represent a point of novelty of the invention (*e.g.*, a nucleic acid that encodes a protein of interest). This general principle, when applied to the present claims, strongly supports Applicants' position that the subject matter of the present claims is more than adequately described.

VI. Rejection under 35 U.S.C. § 112, first paragraph: Enablement

Claims 1-18 and 20-27 were rejected under 35 U.S.C. § 112, first paragraph as allegedly being nonenabled for the full scope for encompassing use of any virus. (Office Action, p. 7-8, ¶¶ 15-17.) The Examiner also asserted that it would take undue experimentation to identify fusion intermediates and antibodies thereto for all claimed viruses. (*Id.*, p. 8, ¶ 16.) Applicants respectfully traverse the rejection as applied to the original or amended claims.

As described in detail in sections V(a) and (b), above, the state of the art concerning viral envelope proteins, fusion intermediates, and antibodies thereto, was extremely high, and the specification provides further guidance for generating conformation-specific antibodies. In particular, the evidence discussed above establishes that viruses share a common mechanism for inducing membrane fusion. Further, an artisan of ordinary skill could accurately predict which sequences of an envelope protein are involved in forming the fusion structure, even for viruses that are not closely related to HIV or influenza. Production of peptides or recombinant proteins that mimic the fusion structure was well within the level of ordinary skill, as was producing and testing antibodies for the desired reactivity.

Given all that was known in the art and all that is taught in the specification, it would have been routine to identify fusion intermediates and to produce antibodies against the intermediates. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

VII. Rejection for Obviousness Type Double Patenting

Claims 1-18 and 20-27 were rejected over claims 1-19 of U.S. Patent No. 6,605,427 ("the '427 patent") under the doctrine of obviousness-type double patenting. (Office Action, pp. 9-11, ¶¶ 18-22.) Applicants respectfully traverse the rejection as applied to the pending claims.

The pending claims are distinct from the claims of the '427 patent because, as explained below, the patent claims require the use of triggering agents, virus permissive cells, or receptors, while the pending claims, as presently amended, require the absence of such reagents.

Claim 1 of the '427 patent recites:

> A method for determining the effect of a test compound on the formation of a conformational intermediate of viral entry and/or fusion, comprising contacting a viral envelope protein or glycoprotein with a *triggering agent* and a candidate compound to form a mixture, and thereafter measuring the effect that the candidate compound has on the formation of said conformational intermediate.

The '427 patent, col. 67. Claim 1 therefore requires contacting a viral envelope protein with a triggering agent in addition to the test compound. Claims 2-4 depend from claim 1, and therefore also require the use of a triggering agent. The term "triggering agent" is described in the '427 patent as an agent that interacts with the envelope protein system and induces entry-relevant conformational changes in the transmembrane protein or fusion protein of the system. 'The '427 patent, col. 9, lines 7-12. According to the '427 patent, triggering agents include virus permissive cells and receptors from those cells. *Id.*, lines 13-18. A "virus permissive cell" is a cell that allows entry into and infection by a particular virus. *Id.*, lines 16-18. As was well-known in the art, cells are permissive when they express a cellular receptor(s) required by that virus. E.g., Eckert and Kim, p. 779, 3d paragraph. Specifically, permissive cells for HIV-1 include lymphocytes, and cellular receptors required for HIV-1 entry include CD4, CCR5, and CXCR4. *See* the '427 patent, col. 9, lines 22-33 (listing lymphocytes and CD4, CCR5, and CXCR4 as triggering agents).

Similarly, claim 5 of the '427 patent and its dependent claims 6-12 and 19, and claim 13 and its dependent claims 14-18, require the use of a virus permissive cell, receptor therefrom, or combination thereof to test the ability of a compound to affect the formation of an entry-relevant structure or conformation necessary for viral entry into a permissive cell.

Therefore, all of the claims of the '427 patent require the use of a triggering agent (e.g., permissive cell or cellular receptor for a virus) that induces conformational changes in a viral envelope protein in a method of determining the effect of a compound on the ability of the envelope protein to form a conformational intermediate or an entry-relevant conformation.

In contrast, pending claim 1 of the present application recites:

> A method for identifying a compound that selectively induces changes in viral envelope proteins that result in loss of function by envelope structures necessary for virus entry, comprising:
>
> providing a membrane bound viral envelope protein or membrane bound fragment thereof,
>
> contacting said membrane bound viral envelope protein or membrane bound fragment thereof with a candidate compound *in the absence of cellular receptors or fragments thereof*; and

> measuring the ability of said candidate compound to induce conformational changes in said membrane bound viral envelope protein or fragment thereof by determining the binding of an antibody, fragment thereof, or peptide to said membrane bound viral envelope protein or fragment thereof.

Claim 1 therefore requires the *absence* of cellular receptors. Likewise, claims 21 and 32 require the *absence* of cellular receptors. Claims 2-9, 14-18, 20, and 22-31 depend from claim 1 or claim 21, and also require the absence of cellular receptors.

Claim 33 depends from claim 32, and further requires incubating the viral envelope protein in the presence of one or more cellular receptors or fragments thereof. However, since claim 33 depends from claim 32, it must be construed such that, regardless of the timing of the incubation step (the step of incubating the viral envelope with the cellular receptor or fragment), this step must be carried out separately from the step of contacting the envelope protein with the test compound. This is in contrast to the claims of the '427 patent, which require mixing together the envelope protein, the triggering agent and the test compound.

Because the claims of the '427 patent require the presence of a triggering agent, a permissive cell, or a cellular receptor, and the claims of the present application require the absence of cellular receptors, the claims of the present application are nonobvious over the patent claims. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

VIII. Rejection under 35 U.S.C. § 103

Claims 1-18 and 20-27 were rejected as allegedly being obvious under 35 U.S.C. § 103 in view of U.S. 6,605,427 ("the '427 patent") in combination with Liang [sic, Jiang]

(1998 and 1999). (Office Action, pp. 11-14, ¶¶ 23-30.) Applicants respectfully traverse the rejection as applied to the pending claims.

The ‘427 patent discloses a method of determining whether a test compound affects the ability of a permissive cell or receptor therefrom to induce the formation of a conformational intermediate in a viral envelope protein. Importantly, the '427 patent states that the "method of the invention *requires* a triggering agent." The '427 patent, col. 9, lines 7-8. Likewise, all of the claims of the '427 patent require contacting or mixing the viral envelope protein with a triggering agent along with a test compound. *Id.*, cols. 67-70. As discussed above (see section VII, above), triggering agents include cells permissive for viral infection and cellular receptors that are required for virus infection. *Id.*, lines 8-39. In addition, none of the test compounds identified by the assay described in the '427 patent *induce* the formation of conformational intermediates in the *absence* of a triggering agent.

In contrast to the disclosure and claims of the '427 patent, the pending claims are directed to a method for identifying compounds that *induce* a conformation in a viral envelope protein. The resulting conformation results in the loss of function by envelope structures required for virus entry. Importantly, the pending claims require the *absence* of cellular receptors or fragments thereof at the time the envelope protein is contacted with the test compound.

The '427 patent does not teach or suggest a method in which an envelope protein is contacted with a test compound in the *absence* of a cellular receptor, and it does not teach or suggest a method to identify compounds that *induce* conformations in envelope proteins. Therefore, the '427 patent does not teach or suggest the claimed invention.

Moreover, neither Jiang (1998) or Jiang (1999) cure the deficiencies of the '427 patent. Therefore, the claimed invention is patentable in view of the '427 patent alone or in combination with Jiang (1998) and Jiang (1999). Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 103.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided. Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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EXHIBIT 1
Appl. No. 10/685,801

using yeasts as potential sources of edible oils, known as single-cell oils, have chiefly centered on the ability of some species such as *Candida curvata* (also referred to *Apiotrichum curvatum*) to produce a facsimile of cocoa butter in which the Δ9-desaturase gene, which converts stearic acid (18:0) to oleic acid (18:1), has been deleted by genetic mutation. Such cells contain up to 50% stearic acid in an overall oil content of about 35 to 40%.

The genetics of lipid synthesis including fatty acid and phospholipid biosynthesis have been studied in detail in *Saccharomyces cerevisiae,* which is not an oleaginous species and therefore does not contain ATP:citrate lyase. Leading investigators include E. Schweizer of Erlangen, Germany, and George M. Carman, Rutgers University, New Jersey.

Filamentous fungi produce a wide range of fatty acids and of lipid types. They may be as diverse as plants in this respect, though many species of fungi (> 60,000 known) have yet to be thoroughly examined for their lipid composition. No systematic survey of fungal lipids has been carried out, and there is still much waiting to be discovered. Little attempt has been made to use lipid components as chemotaxonomic markers, and very little work has been carried out on the genetics or molecular biology of fungal lipids. In some cases, there may be extensive accumulation of triacylglycerols as storage reserves, and oil contents of up to 85% have been recorded. These are also referred to as oleaginous microorganisms.

One of the few differences between the major families of the molds is that members of the lower fungi Phycomycetes (i.e., the orders Mastigomycotina and Zygomycotina, which encompasses 1935 species) produce γ-linolenic acid (GLA) [18:3(6,9,12)] rather than the α-isomer [18:3(9,12,15)] found in all other fungi comprising the slime fungi (Myxomycota) and the higher fungi of the order Ascomycetes (Ascomycotina), Basidiomycetes (Basidiomycotina), and the fungi imperfecti (Deuteromycotina).

The use of fungi to produce GLA and, more recently, dihomo-GLA (20:3), arachidonic acid (20:4), and the highly polyunsaturated fatty acids eicosapentaenoic acid (20:5) (EPA) and docosahexaenoic acid (22:6) (DHA), has been of some commercial interest because of the dietetic importance of these acids. Processes for the production of oils containing each of these fatty acids have been described and fungal oils with GLA have been produced commercially both in the United Kingdom and in Japan in recent years. As the enzyme for the formation of GLA from 18:2 (i.e., Δ6-desaturase) occurs in only a few plants (evening primrose, borage, and *Ribes* spp.), it may be that the molds that produce GLA (*Mucor, Mortierella, Pythium, Rhizopus,* etc.) could be used as a source of the Δ6-desaturase gene. This could be inserted into a plant, such as linseed, which already produces α-linolenic acid, so that if the Δ15-desaturase activity could be removed, the plant would now produce GLA instead of the α-isomer. These fungi appear to be genetically manipulatable, and other aspects of the physiology of *Mucor* spp. have been genetically explored.

4 UNICELLULAR ALGAE

Like plants, algae contain a wide variety of lipid types, many of which are intimately associated with photosynthesis. The lipids of the prokaryotic algae, the cyanobacteria (*Anabaena, Anacystis, Oscillatoria, Synechococcus,* etc.), contain a diversity of lipid types and are distinguished from other prokaryotic phototrophs by containing plant chlorophyll rather than bacteriochlorophyll. Unlike their bacterial counterparts, cyanobacteria synthesize polyunsaturated fatty acids, with some producing γ-linolenic acid while other produce the α-isomer. These, and other fatty acyl groups, are associated with complex lipids such as a quinovosyl sulfolipid (= "plant sulfolipid") mono- and digalactosyldiacylglycerol and phosphatidylglycerol. Some triacylglycerols may occur as storage material.

Eukaryotic algae can be divided into the freshwater and marine varieties. The former tend to produce fatty acids with a much lower degree of unsaturation than the latter.

Organisms that have been studied for their potential as lipid producers include the halophile *Dunaliella salina,* which accumulates β-carotene and has been grown commercially in the sea and in hypersaline ponds in Western Australia, Isarel, and California. Interest now focuses on species of *Spirulina* and *Porphyridium* (a red alga) as sources of GLA and EPA. Some species can be grown heterophotically in the dark (i.e., using sugar as a carbon and energy source) to produce either EPA or DHA.

Recombinant DNA technologies are now being successfully applied to the green alga *Chlorella ellipsoidea,* to improve production of selected lipids and other products as well. However, this work is very much in its infancy, and a long-term strategy will be needed if success is to be achieved.

See also LIPIDS, STRUCTURE AND BIOCHEMISTRY OF; LIPOSOMAL VECTORS; PHOSPHOLIPIDS.

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LIPIDS, STRUCTURE AND BIOCHEMISTRY OF

Donald M. Small

Key Word

Lipid Any molecule of intermediate molecular weight (100–5000) that contains a substantial portion of aliphatic or aromatic hydrocarbon.

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The major chemical classes of lipids (Table 1) include substituted hydrocarbons, esters of fatty alcohols and fatty acids, and complex lipids such as acylglycerols, phospholipids, glycoglycerolipids, sphingolipids, steroids, and vitamins. Lipids are found in all living organisms and function primarily as barriers between the cell and the environment and between compartments within the cell. They have specific functions as receptors, sensors, electrical insulators, antigens, biological detergents, and membrane anchors for proteins. Membranes are made of lipids capable of forming a barrier between two aqueous compartments. The membrane-forming lipids include phospholipids, glycolipids, and sphingolipids, whereas oils, fats, and waxes are major sources of stored fatty acids and cellular energy. Most lipids are composed of substituted alkanes, specifically fatty acids, fatty alcohols, or sphingosine, linked to a variety of other chemical groups. For instance, Figure 1 shows the general structure of a glycerol phospholipid. Fatty acids are esterified to the 1 and 2 positions of glycerol and phosphate to the 3 position. The common phospholipids have different bases (choline, ethanolamine, serine, glycerol), which are esterified to the phosphate group.

Table 1 A Chemical Classification of Lipids[a]

I. Hydrocarbons (normal, branched, saturated, unsaturated, cyclic, aromatic)
II. Substituted hydrocarbons
  A. Alcohols
  B. Aldehydes
  C. Fatty acids, soaps, acid-soaps
  D. Amines
III. Waxes and other simple esters of fatty acids
IV. Fats and most oils (esters of fatty acid with glycerol)
  A. Triacylglycerols
  B. Diacylglycerols
  C. Monoacylglycerols
V. Glycerophospholipids (diacyl, O-alkyl, acyl, di-O-alkyl)
  A. Phosphatidic acid
  B. Choline glycerophospholipids
  C. Ethanolamine glycerophospholipids
  D. Serine glycerophospholipids
  E. Inositol glycerophospholipids
  F. Phosphatidylglycerols
  G. Lysoglycerophospholipids
VI. Glycoglycerolipids, including sulfates
VII. Sphingolipids
  A. Sphingosine
  B. Ceramide
  C. Sphingomyelin
  D. Glycosphingolipids [ceramide monohexosides (cerebrosides), ceramide monohexosides sulfates, ceramide polyhexosides]
  E. Sialoglycosphingolipids (gangliosides)
VIII. Steroids (sterols, bile acids, cardiac glycosides, sex and adrenal hormones)
IX. Other lipids [vitamins (A, D, E, K), eicosanoids, acyl CoA, acyl carnitine, glycosyl phosphatidylinositols, lipopolysaccharides, dolichols]

[a] This chemical classification of lipids is necessarily incomplete and somewhat arbitrary. It progresses from hydrocarbons to more complex chemical structures. Simple esters and glycerol esters yield on hydrolysis the alcohol and/or glycerol and fatty acid. The major membrane lipids, glycerophospholipids, yield fatty acid (or alcohol), glycerol, phosphate, and the appropriate base (choline, ethanolamine, etc.). Sphingolipids yield the base sphingosine and a fatty acid on hydrolysis.

Source: D. M. Small and R. A. Zoeller, Lipids. In *Encyclopedia of Human Biology,* Vol. 4. Academic Press, San Diego, CA, 1991.

Phospholipids are amphiphilic (see Figure 2); that is, they have a hydrocarbon part which is soluble in oil (lipophilic) and a water-soluble part (hydrophilic)—for instance, phosphorylcholine. Thus, the molecules can partition between the oil–water interface with the hydrocarbon chains in the oily side of the interface and the water soluble polar groups in the aqueous side. As such, they lower the interfacial tension to form stable interfaces between oil and water which, without these interfacial molecules, would separate from each other. These molecules form the interfaces between globules of nonpolar lipid molecules occurring in cells, such as in adipose tissue cells. In the plasma lipoproteins they form the surface around an oily droplet of triglycerides or cholesterol esters to form a stable interface between plasma and the hydrocarbon core of the lipoprotein. Certain lipids can self-associate so that the hydrophobic parts of the molecules interact and form lamellae, with the polar groups facing toward the water and the hydrocarbon parts interacting with each other. These membranes or bilayers make effective electrical and physical barriers only a few nanometers thick. These lipids are particularly important in cells in that they form the barriers between the external surface of the cell and the cytoplasm (the plasma membrane) and allow the cell to be compartmentalized into its various organelles (mitochondria, microsomes, Golgi apparatus, nuclear membranes, secretory vesicles, etc.). In most membranes the lipids are in the liquid state. That is, the chains are melted like oil. The chains wobble on a nanosecond time scale, move laterally one molecule width (~ 8 A) every 10 to 15 picoseconds, but take days to flip from one side to the other. Under the right circumstances bilayers form closed structures (vesicles), which separate an internal aqueous phase from the surrounding one. Vesicles can be used to trap drugs or DNA and can be targeted to certain cells for specific uptake. When some viruses bud from cells they are encapsulated by a vesicle composed of the plasma membranes of the cell. Of course much transport within cells is carried out by vesicles budding from one organelle, moving to another, and fusing with it. Other lipid molecules with particularly large or highly charged polar groups relative to their hydrocarbon region spontaneously form small spherical, cylindrical, or disklike aggregates in aqueous systems. The molecules of these small aggregates are in rapid equilibrium with a low concentration of monomers in aqueous phase. Such aggregates, called micelles, are capable of solubilizing many other kinds of hydrophobic molecule by dissolving them in the hydrocarbon core of the micelle. The concentration of monomer molecules in the aqueous phase is called the critical micellar concentration (cmc) because when the concentration of lipid increases above the cmc, micelles begin to form.

From the chemical classification of lipids in Table 1, it is impossible to predict what the physical behavior will be like. For instance, both fatty acids and their alkali soaps are classified as substituted hydrocarbons; however fatty acids are largely insoluble in aqueous systems, whereas soaps are quite soluble and form micelles. Thus, to understand how lipids behave within cells and within biological fluids, we have devised a classification based on the behavior of lipids in water and at the aqueous interface (Table 2). The classification applies to lipids with melted chains, since most biological lipids within cells appear to have the liquid chain conformation. The lipids are classified as nonpolar or polar. Nonpolar lipids have extremely low aqueous solubility and do not spread at the air–water or oil–water interface to form a monolayer. That is, they

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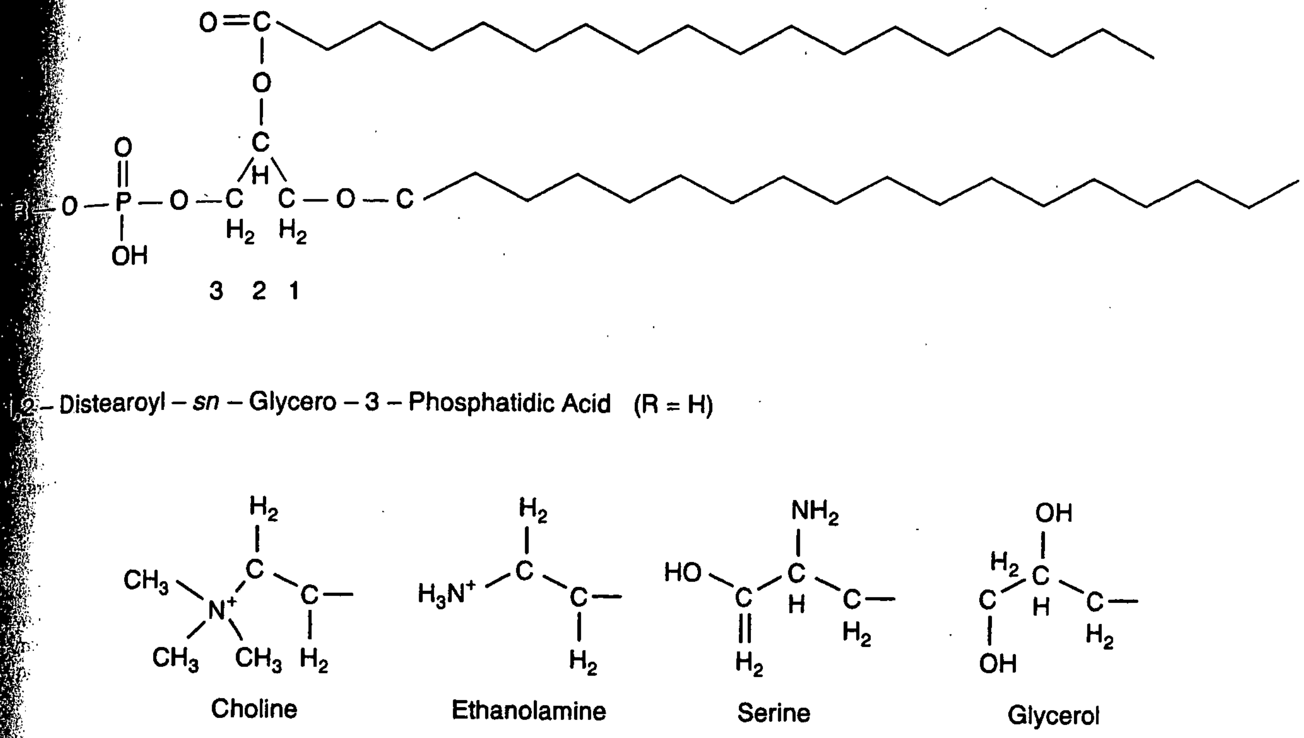

Figure 1. Line models of some phospholipids. If R is hydrogen, the compound is phosphatidic acid as indicated. If the polar moieties shown are substituted for R, the compound is the corresponding phosphatidyl-R (i.e., if R is choline, the compound is phosphatidylcholine. (From D. M. Small and R. A. Zoeller, Lipids. In *Encyclopedia of Human Biology,* Vol. 4, Academic Press, San Diego, CA, 1991.)

do not have bulk or surface solubility. Class I polar lipids have surface solubility but no bulk solubility. They can partition into membranes but cannot form membranes (bilayers) by themselves. Class II lipids (i.e., the phospholipids and glycosphingolipids) are the membrane formers. Class III lipids (i.e., detergents) (are soluble lipids that form micelles. Monolayers of these lipids are unstable. Molecules on the surface and in micelles are in constant rapid equilibrium with molecules in the aqueous phase. Low concentrations of these molecules can penetrate into bilayers and membranes but above a certain concentration will cause disruption, and high concentrations will dissolve membrane lipids into micelles. They also bind to and alter the conformation of proteins. Fortunately, except for a few specific cases (e.g., bile salts in bile and intestinal contents), they are in low concentration, well below their critical micellar concentration, and usually are partitioned within membranes or lipoproteins or bound to specific proteins like albumin.

1 SEPARATION, EXTRACTION AND ANALYSIS OF LIPIDS

Lipids are present in a variety of organelles within cells and in extracellular fluids such as blood plasma, bile, milk, and intestinal contents. First, the particular compartment to be characterized (e.g., red blood cells or low density lipoproteins or mitochondria) is isolated. It is then extracted with chloroform/methanol (2:1, v/v). Sufficient chloroform/methanol is required to form a single phase in which the tissue water is dissolved. Insoluble precipitates form, and these materials which include proteins, nucleic acids and polysaccharides, are removed by centrifugation or filtration. Excess water containing acid and salts is then added to form a two-phase system: water/methanol on top and chloroform on the bottom. The lipid partitions into the chloroform phase, which is removed and concentrated by evaporation, and an aliquot is dried to obtain the weight. Lipids are then subjected to high performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) to separate them into the major classes—for example, hydrocarbons, wax esters, sterol esters, triacylglycerols, fatty acids, sterols, and phospholipids. Then each individual lipid class is collected and rechromatographed on a different system to isolate the individual components. For instance, phospholipids would be separated into phosphatidylcholines, sphingomyelins, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, and so on. Each individual type of phospholipid—for instance, phosphatidylcholine—can be derivatized and separated on a new HPLC or gas–liquid chromatographic (GLC) column, to discriminate between the different acyl chains on each phosphatidylcholine. These individual peaks are then examined by mass spectrometry or nuclear magnetic resonance techniques to define the chemical structure. The process is long and involved and requires some modifications for each different lipid class. For instance, while the individual molecular species of phosphatidylcholine have been fully characterized for several lipoproteins and even bile, the different molecular species of triacylglycerols are so complex that not even butter fat, which is a highly studied lipid, has been totally dissected into all its individual triacylglycerols. A common short cut is to isolate the individual lipid class (e.g., phosphatidylcholines or phosphatidylserine), then hydrolyze the fatty acids and analyze them by GLC. This procedure gives the overall composition of fatty acids on the specific class but not the molecular species of the individual phospholipids.

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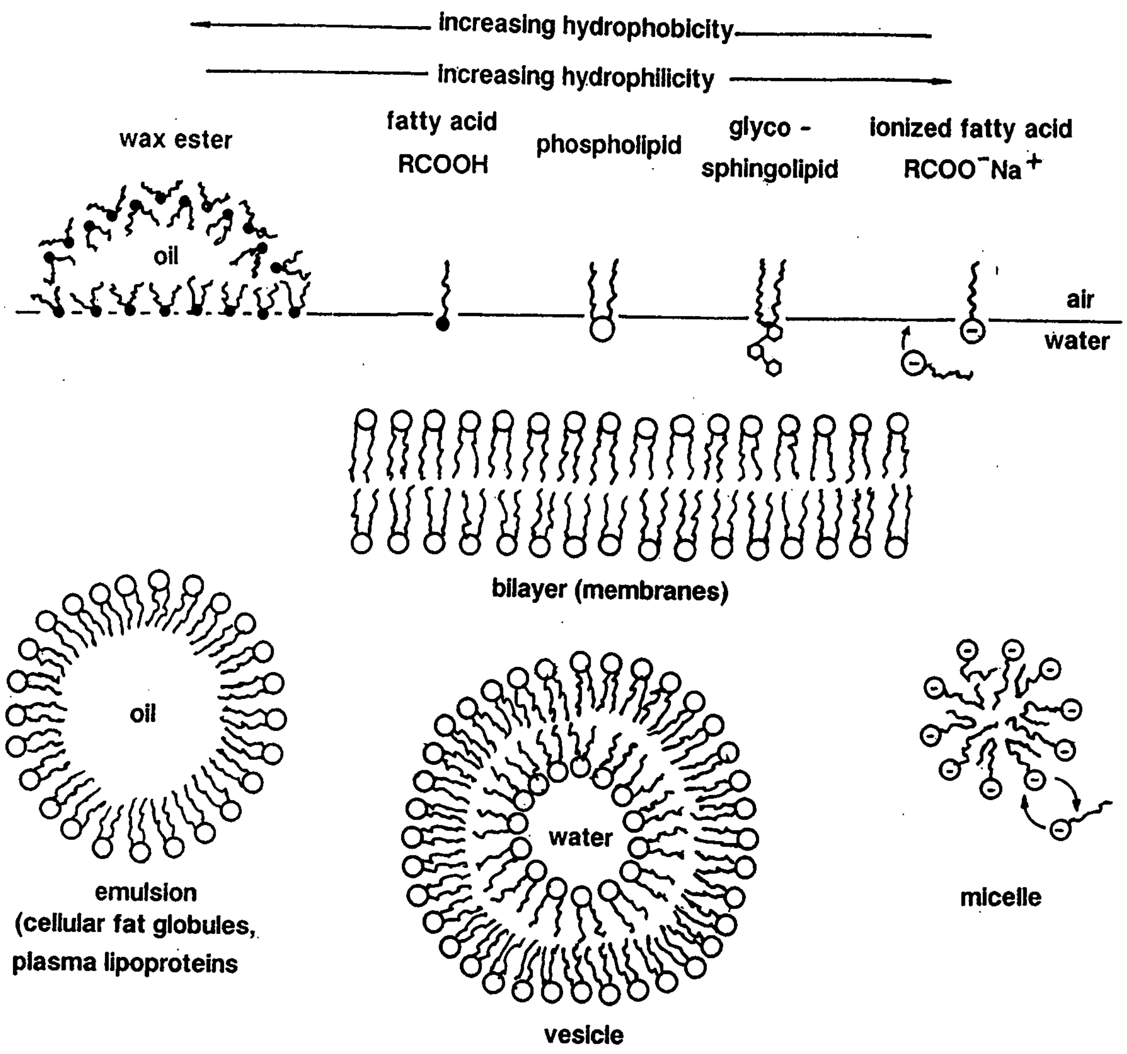

Figure 2. Common structures formed by lipids at an air–water interface and in aqueous systems. The upper part of the diagram indicates the behavior of lipids of increasing hydrophilicity (or decreasing hydrophobicity) at an air–water interface. Nonpolar lipids (left) such as wax esters or long chain alkanes form drops of oil that do not spread and simply sit as a lens on the surface of water. They are soluble in organic solvents. The polar lipids (e.g., protonated fatty acids, phospholipids, glycosphingolipids) form stable monolayers. Phospholipids and glycosphingolipids are capable of forming bilayers (membranes) and of stabilizing nonpolar lipids in the center of emulsion droplets by providing the surface with a monolayer of amphiphilic lipid. Ionized fatty acids (epg., sodium or potassium soaps) are detergents and are soluble in water (and insoluble in organic solvents like hexane or benzene); they desorb from the surface rapidly. Above a critical aqueous concentration (the critical micellar concentration, cmc) little aggregates form which are called micelles. The cmc for sodium oleate is about 1.5 mM. High concentrations of detergents can dissolve membranes and solubilize membrane proteins. Fortunately detergents rarely occur physiologically in high enough concentration to damage membranes. Phospholipids and glycosphingolipids membranes can be made to form closed vesicles with an aqueous cavity in the center. Other material such as drugs or DNA may be encapsulated in this space. Ligands for particular receptors may be adsorbed to the surface of it to direct the vesicle to specific cell types, where it is recognized and taken up into the cell.

2 OCCURRENCE AND METABOLISM

Hydrocarbons contain carbon and hydrogen and can be saturated or unsaturated, branched or cyclic. The source of most hydrocarbons is petroleum. Alkanes can be metabolized in very limited amounts by mammalian cells by converting the alkane to a normal alcohol, which may be further oxidized to fatty acids. Polymeric compounds such as the 40-carbon compound carotene can be split into two 20-carbon vitamin A alcohols in the intestine. The 30-carbon polyisoprenoid squalene, which is synthesized in the liver, can be oxidized by specific enzymes and cyclized to form cholesterol and other steroids. Cholesterol is a precursor of bile acids and adrenal and sex hormones. Other enzyme systems, which are different from those that oxidize alkanes to alcohols, can oxidize and detoxify aromatic hydrocarbons such as benzpyrene.

The predominant class of *substituted hydrocarbons* is the fatty acids (RCOOH), but alcohols (ROH) and aldehydes (RCO) are also found in low concentrations. The major fatty acids (Table 3) consist of saturated, monounsaturated, and polyunsaturated fatty acids that vary in chain length and in double-bond position. Most fatty acids can be synthesized by mammalian cells; however in some mammals linoleic and linolenic acids cannot be synthesized de novo and must be ingested in the diet, hence qualify as essential fatty acids. Linoleic acid is the precursor of arachidonic acid, which is the major source of eicosanoids. Linolenic acid is a precursor for eicosapentenoic and docosahexenoic acids, which form complex

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Table 2 Physical Classification of Biologically Active Lipids

Class[a]	Surface Properties	Bulk Properties	Examples
Nonpolar	Will not spread to form monolayer	Insoluble	Long chain, saturated or unsaturated, branched or unbranched, aliphatic hydrocarbons with or without aromatic groups (epg., dodecane, octadecane, hexadecane, paraffin oil, phytane, pristane, carotene, lycopene, gadusene, squalene) Large aromatic hydrocarbons (epg., cholestane, benzpyrenes, coprostane, benzphenanthrocenes) Esters and ethers in which both components are large hydrophobic lipids (epg., sterol esters of long chain fatty acids, waxes of long chain fatty acids, and long chain normal monoalcohols, ethers of long chain alcohols, sterol ethers, long chain triethers of glycerol)
Polar Class I: Insoluble nonswelling amphiphiles	Spreads to form stable monolayer	Insoluble or solubility very low	Triglycerides, diglycerides, long chain protonated fatty acids, long chain normal alcohols, long chain normal amines, long chain aldehydes, phytols, retinols, vitamin A, vitamin K, vitamin E, cholesterol, desmosterol, sitosterol, vitamin D, un-ionized phosphatidic acid, sterol esters of very short chain acids, waxes in which either acid or alcohol moiety is less than 4 carbon atoms long (epg., methyl oleate), ceremides
Class II: Insoluble swelling amphipathic lipids	Spreads to form stable monolayer	Insoluble but swells in water to form lyotropic liquid crystals	Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, cardiolipid, plasmalogens, ionized phosphatidic acid, cerebrosides, phosphatidylserine, monoglycerides, acid-soaps, α-hydroxy fatty acids, monoethers of glycerol, mixtures of phospholipids and glycolipids extracted from cell membranes or cellular organelles (plant glycolipids and sulfolipids) sulfocerebrosides, sphingosine (basic form)
Class IIIA: Soluble amphiphiles with lyotropic mesomorphism	Spreads but forms unstable monolayer because of solubility in aqueous substrate	Soluble, forms micelles above a cmc; at low water concentrations forms liquid crystals	Sodium and potassium salts of long chain fatty acids, many of the ordinary anionic, cationic, and nonionic detergents, lysolecithin, palmitoyl and oleyl coenzyme A, and other long chain thioesters of coenzyme A, gangliosides, sphingosine (acid form)
Class IIIB: Soluble amphiphiles, no lyotropic mesomorphism	Spreads but forms unstable monolayer because of solubility in aqueous substrate	Forms micelles but not liquid crystals	Conjugated and free bile salts, sulfated bile alcohols, sodium salt of fusidic acid, rosin soaps, saponins, sodium salt of phenanthrenesulfonic acid, penicillins, phenothiazines

[a] Lyotropic mesomorphism means the formation of liquid crystals (epg., bilayers or other lipid aggregates) on interaction with water.

Source: D. M. Small, The physical chemistry of lipids from alkanes to phospholipids. In *Handbook of Lipid Research,* Vol. 4, D. Hanahan, Ed. Plenum Press, New York, 1986.

membrane lipids in the retina and brain. Fatty acids are synthesized de novo from acetate. Acetyl CoA carboxylase and the multifunctional polypeptide *fatty acid synthetase* work together to elongate the chain by two carbons at each step to produce long chain fatty acids, such as palmitic and stearic acids. The chain may be elongated further by fatty acyl CoA elongation system, which adds two carbon fragments. The chain may be desaturated to form a double bond by specific desaturases such as the *Δ9-desaturase,* which forms oleic acid from stearic acid.

Fatty acids are broken down in two carbon units to yield acetyl CoA. The process is called *beta oxidation* and yields energy with each acetyl CoA molecule formed. Very long chain fatty acids are broken down in peroxisomes and short chain fatty acids are broken down in mitochondria. Normal long chain fatty acids like palmitic and stearic acid can be broken down by either organelle. Fatty acids may also be obtained from diet, where they are present as components of dietary fats and oils.

Dietary fatty acids are transported into the intestinal cell, synthesized into *triglycerides* and assembled into chylomicrons, and secreted into the circulation. There the triglyceride of the chylomicron is hydrolyzed by lipoprotein lipase and the fatty acid distributed to tissues in the capillaries and to circulating albumin. The albumin carries fatty acid back to the liver, where it is rapidly taken up and distributed in the cell. Both newly synthesized fatty acid and fatty acid arriving from the plasma either are catabolized through beta oxidation to produce energy or are incorporated into other lipids such as phospholipids or triacylglycerols or other esters. Strictly speaking protonated fatty acid is a class I polar lipid: it binds readily to albumin and in the cells may distribute between membranes and intracellular fatty acid binding proteins. In membranes, however, it is about half-ionized and contributes a net charge to membranes.

When the cellular uptake of fatty acids or its synthesis exceeds the catabolism of fatty acids, the excess must be stored. Plankton and many lower aquatic animals store *wax esters.* The reaction apparently involves an enzyme that utilizes acyl coenzyme A and free alcohol. Waxes are nonpolar molecules and form the cores of storage droplets in both plants and lower animals. In many higher animals triacylglycerols, not wax esters, are the major form of storage lipid.

Triacylglycerols are formed by two general pathways. The first utilizes phosphatidic acid. It is converted to 1,2-*sn*-glycerol by removal of the phosphate group and then acylated to triacylglycerol

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Table 3 Names, Formulas, and Selected Properties of Common Fatty Acids

Fatty Acid	Chemical Name	Δ Formula[a]	ω Formula[b]	Molecular Weight	Melting Point (°C)	Solubility in Water at 25°C (μM)
	Saturated Fatty Acids					
Lauric	Dodecanoic acid	12.0		200.31	44.2	11.5
Myristic	Tetradecanoic acid	14.0		228.36	54.4	7.9×10^{-1}
Palmitic	Hexadecanoic acid	16.0		256.42	62.9	1.2×10^{-1}
Stearic	Octadecanoic acid	18.0		284.47	69.6	1.8×10^{-2}[c]
	Monounsaturated Fatty Acids					
Palmitoleic	*cis*-9-Hexadecenoic acid	16.1 9C	*c*16:1ω7	254.40	0.5	3×10^{-1}[c]
Oleic	*cis*-9-Octodecenoic acid	18.1 9C	*c*18:1ω9	282.45	13.4α16.3β	4.5×10^{-2}[c]
	Polyunsaturated Fatty Acids					
Linoleic	*cis*-9,12-Octadecadienoic acid	18.2 9C12C	*c*18:2ω6	280.44	−5	
Linolenic	*cis*-9,12,15-Octadecatrienoic acid	18.3 9C12C15C	*c*18:3ω3	278.44	−10(−11.3)	
Arachidonic	*cis*-5,8,11,14-Eicosatetraenoic acid	20.4 5C8C11C14C	*c*20:4ω6	304.5	−49.5	
Eicosapentaenoic[d]	*cis*-5,8,11,14,17-Eicosapentaenoic acid	20.5 5C8C11C14C17C	*c*20:5ω3	302.5		
Docosahexaenoic[d]	*cis*-4,7,10,13,16,19-Docosahexaenoic acid	22.6 4C7C10C13C16C19C	*c*22.6ω3	328.5		

[a] Formulas are shown as the number of carbon atoms followed by the number of double bonds. The position of each double bond is indicated by the lower of the numbers of the two doubly bonded carbon atoms, counting from the carboxyl carbon and specified as to *cis* (C) or *trans* (T) configuration. Thus, oleic (*cis*-9-octadecenoic) acid is 18.1 9C.

[b] The numbering of the carbon atoms starts from the methyl end, and the location of the first (or only) double bond is indicated by a single number as a suffix preceded by "ω." Oleic acid is therefore designated as *c*18:1ω9, which informs the reader that the most distal double bond is nine carbons from the methyl terminus.

[c] Extrapolated from solubility of shorter chain acids.

[d] Found in marine animals in high concentration and also in retina and brain of mammals.

with an activated fatty acid by *acyl CoA diacylglycerol acyltransferase.* A second pathway involves the reacylation of 2-acyl-*sn*-glycerol, first by *monoacylglycerol acyltransferase,* to form the diglyceride, and then by *diacylglycerol acyltransferase,* to form triacylglycerol. This pathway is prominent in the intestine and is utilized to form the triglyceride, which is transported from the intestine in chylomicrons.

Triacylglycerols are broken down in many places by lipases. In the intestinal lumen, dietary fat is broken down by pancreatic lipases to two moles of fatty acid and one mole of 2-acyl-*sn*-glycerol (monoglyceride). In plasma triglycerides are broken down by *lipoproteins lipase* to the same molecules, which then partition into tissues or are transferred to albumin. In cells the triacylglycerol storage droplets are broken down by *hormone-sensitive lipase,* which produces fatty acid and glycerol in the cell.

The phospholipids shown in Figure 1 are made through several pathways involving either *phosphatidic acid* or *diacylglycerol.* Phosphatidic acid may be produced by the action of phospholipase D on more complex phospholipids or, more commonly, by the acylation of glycerol 3-phosphate with two moles of activated fatty acid to form phosphatidic acid. This key molecule may be hydrolyzed to form diacylglycerol, which can condense with cytosine diphosphate (CDP) choline to form phosphatidylcholine. Phosphatidic acid can interact with cytosine triphosphate to form cytosine diphosphate diacylglycerol (CDP-diglyceride). This intermediate can condense with serine to form phosphatidylserine. Phosphatidylserine may be decarboxylated to form phosphatidylethanolamine, a major membrane lipid.

A second set of reactions sequentially adds methyl groups to phosphatidylethanolamine to produce mono- and dimethylethanolamine and finally phosphatidylcholine. Phospholipids are subject to a variety of phospholipases, which hydrolyze the different ester and ether bonds to break down the phospholipids into various individual groups (fatty acids, glycerol, choline, ethanolamine, etc.), which enter the general metabolic pool. A separate set of reactions forms alkyl (ether) bonds between alcohols and glycerol. Ethers tend to be more stable than esters, and vinyl ether phospholipids such as plasmalogens may play important roles in stabilizing membranes in oxidatively reactive compartments such as lysosomes and peroxisomes.

Glycosphingolipids are formed by the addition of sugars to *N*-acylsphingosine (ceramide). In a complex set of reactions, different sugar moieties are added to ceramide to produce complex glycosphingolipids. This general class of molecules is found in most cells. Some can act as receptors for a variety of toxins and as antigens for antibodies. Others perhaps act as cell-type labels. They are particularly prominent in the brain and nerves, where they form a major portion of the membrane systems. These complex molecules are broken down by glycosyl hydrolases, which catalyze the specific removal of one sugar at a time, ultimately reducing the complex glycosphingolipids to ceramides and sugars. Deficiencies of enzymes along this pathway result in the accumulation of specific glycosphingolipids, and serious neurological disorders accompany these metabolic blocks. For instance the inability to break down gangliosides results in Tay–Sachs disease, and the inability to break down galactocerebroside, which has only one sugar on the ceramide, results in Gaucher disease. The symptoms of these diseases may arise from the physical properties of the lipids. For instance, gangliosides tend to accumulate in Tay–Sachs disease and cause massive swelling and disruption of membrane within the cells. Certainly part of their effect is due to the detergent properties of the accumulating gangliosides. While genetic defects

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lipid metabolism or catabolism lead to a wide variety of disease states; many serious diseases involving lipids (e.g., atherosclerosis, obesity, cholesterol gallstone disease) may result from play between the environment and as yet unrecognized genetic factors.

See also LIPID METABOLISM AND TRANSPORT; LIPIDS, MICROBIAL; LIPOPROTEIN ANALYSIS; LIPOSOMAL VECTORS; PHOSPHOLIPIDS; PLASMA LIPOPROTEINS; STEROID HORMONES AND RECEPTORS.

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LIPOPOLYSACCHARIDES

Ilkka M. Helander, P. Helena Mäkelä, Otto Westphal, and Ernst T. Rietschel

Key Words

Endotoxicity The acute effects of lipopolysaccharide (endotoxin) on animals.

Endotoxin Synonym for lipopolysaccharide, used when referring to the biological activity of LPS; distinct from bacterial exotoxins, which are proteins secreted from cells.

Gram-Negative Bacteria Category of bacteria with a cell envelope containing an outer membrane with lipopolysaccharide as an essential component; this property is associated with the negative (red) staining of the cells in the selective Gram staining method.

Lipid A The lipid part of a lipopolysaccharide, which is responsible for its endotoxic activities.

O-Antigen Synonym for lipopolysaccharide and especially its outermost polysaccharide region showing specific antigenic reactivity.

Lipopolysaccharides (LPS) are unique structural components of the Gram-negative cell envelope. They exist ubiquitously in all Gram-negative bacteria, constituting an essential component of the outer leaflet of the asymmetrical outer membrane (OM) and providing the membrane and thus the bacterial cell with a hydrophilic surface. Therefore the OM can serve as an effective permeability barrier to many agents, such as hydrophobic antibiotics, detergents, dyes, and bile acids. Besides its functional role in the bacterial cell envelope, a multitude of biological, often toxic effects are evoked in mammalian hosts by LPS released from multiplying or disintegrating bacteria. These effects are referred to as endotoxic activities, and the term "endotoxin" is therefore often used interchangeably with LPS.

1 STRUCTURE AND FUNCTIONS OF LIPOPOLYSACCHARIDES

1.1 GENERAL

Figure 1 shows lipopolysaccharides (LPS) in relation to other structural components of a cell envelope, and Figure 2 shows the schematic structure of a complete LPS molecule of *Salmonella*. In general, LPS consists of a complex lipid, termed lipid A, and a covalently linked heteropolysaccharide. The latter is divided into a core oligosaccharide and an O-specific chain, which is built of repeating oligosaccharide units. In certain bacterial groups (e.g. *Neisseria* spp.) the O-specific chain is lacking. Each part of the LPS molecule is endowed with specific biological and physiological functions.

1.2 LIPID A

The lipid A component consists most commonly of a β1′-6-linked D-glucosamine (GlcN) disaccharide, carrying at positions 1 and 4′ phosphate groups, which are often partially substituted by charged groups. This lipid A backbone carries four *(R)*-3-hydroxy fatty acids in amide linkage at positions 2 and 2′ and in ester linkage at positions 3 and 3′ (see Figure 2). The hydroxyl groups of some of the fatty acids are further esterified with nonhydroxylated fatty acids, creating the unique 3-acyloxyacyl structure. This structural principle is shared by the lipid A from many bacterial groups. In lipid A of different bacterial origin, however, the type and chain length of fatty acids vary greatly, and in some lipid A types, GlcN is replaced by 2,3-diamino-2,3-dideoxy-D-glucose. Lipid A is responsible for the endotoxic activities attributed to LPS. Lipid A variants with essential deviations from the foregoing structure exhibit reduced endotoxicity. In the bacterium, lipid A anchors the LPS molecule in the OM and is absolutely essential for the survival of the bacterium.

1.3 THE POLYSACCHARIDE

The core oligosaccharide is linked to lipid A via the acidic eight-carbon sugar Kdo (3-deoxyoctulosonic acid), often present as a di- or trisaccharide. The core itself is subdivided into an inner

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EXHIBIT 2
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ular design techniques, including the definition of quantitative structure–activity relationships (QSAR) and the calculation of electronic distribution, are increasingly employed to refine early empirical and intuitive approaches. Molecular graphic and docking procedures are widely employed, using experimentally determined structural coordinates if available, or calculated minimum energy conformations (Figure 5). Drug–receptor interactions are, almost without exception, stereoselective. The issue of enantioselectivity is of importance to drug development, which increasingly will focus on chiral compounds. Thus the appropriate stereochemistry must be incorporated at an early stage of structure development and synthesis.

Lead structures are frequently peptides or peptide-containing species because these are natural products or endogenous physiological regulators. Peptides present major problems of drug delivery and stability, and peptidomimetic analogue design is a major issue in lead development. The benzodiazepine ring system may represent one such peptidomimetic, since it provides a nucleus for drugs active at several peptide-served receptors including those for cholecystokinin, gastrin, and opiates.

The generation of new structures that serve as leads or develop existing leads is time-consuming and costly. Several methods have been devised to accelerate and automate this process, and these permit rapid construction of peptide and nucleotide libraries. Such combinatorial libraries may then be screened with receptor molecules and the "active" sequence identified. These combinatorial products can also be obtained by biological methods in which, for example, a repertoire of random oligonucleotides is inserted into a phage vector and each sequence expressed in one phage. These powerful and sensitive genetic methods provide for rapid enrichment and decoding of active sequences. They are, however, limited to nucleotides and naturally occurring amino acids. New developments will extend this approach to additional building blocks and may ultimately incorporate "self-designing" evolutionary chemistry to optimize drug design.

4 PERSPECTIVE

Medicinal chemistry is a rapidly changing discipline. Other physical and biological disciplines have contributed to the increasingly "logical" or "rational" process of drug discovery. Molecular biology has had significant impact by defining target sites and structures and by accelerating, in select fields, the very process of drug synthesis. It is likely that the contributions of medicinal chemistry will become increasingly quantitative in the next decade.

See also Biotransformations of Drugs and Chemicals; Drug Synthesis; Receptor Biochemistry.

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Melting: *see* Denaturation of DNA.

MEMBRANE FUSION, MOLECULAR MECHANISM OF

Koert N. J. Burger

Key Words

Fusogenic Protein Protein directly responsible for the induction of membrane fusion. Also known as fusion protein.

Local Point Fusion Membrane fusion as observed using modern (fast-freeze) electron microscopical techniques: initially restricted to a small area (< 20 nm) of the interacting membranes.

Membrane Fusion Complete coalescence of two membranes leading to the formation of one membrane-enclosed compartment out of two originally separated compartments (as in cell–cell fusion), or two membrane-enclosed compartments out of one (as in cell division).

Type II Lipids Class of fusogenic lipids capable of forming inverted nonbilayer lipid structures.

Membrane fusion is a ubiquitous event in the functioning of a living organism. Life starts as a sperm fuses with an oocyte, leading to its fertilization. Membrane fusion is crucial for the formation of the mature muscle fiber, for exocytosis and endocytosis, and for intracellular membrane traffic. Membrane fusion is used by enveloped viruses to enter and infect cells. Membrane fusion is also an important tool in research and medication. Artificially induced cell fusion is an essential step in the production of monoclonal antibodies, and directed fusion of membrane vesicles may be used to deliver probe molecules and drugs to cells, both in vitro and in vivo.

1 METHODS AND MODEL SYSTEMS

Thousands of membrane fusion events occur in any living cell every minute. Considering that even one mistake (e.g., the fusion of an endosome with the nuclear envelope) would be fatal to the cell, it is clear that membrane fusion occurs only under strict control: specific proteins determine exactly where and when mem-

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brane fusion takes place. Biomembrane fusion is a multistep process of high complexity, and the actual fusion event (membrane coalescence and pore formation) is extremely fast—in the millisecond range. Only by using appropriate model systems can the molecular mechanism of membrane fusion be unraveled. Biochemical approaches using permeabilized cells and cell-free systems composed of isolated intracellular membranes have identified many of the (protein) factors required for membrane fusion. Molecular genetics has confirmed their importance in the regulation of intracellular membrane traffic. Many of the protein factors are universal, occurring both in yeast and in mammalian cells; and similar proteins are involved in many different membrane fusion events within the cell. However, for none of these factors has a direct role in the induction of membrane fusion been shown. The mechanism by which biomembranes are destabilized and induced to fuse remains largely obscure. Because membrane fusion ultimately requires the coalescence of two lipid bilayers, pure lipid systems have been studied extensively. Our current understanding of the molecular mechanisms of membrane fusion is mainly based on results obtained using these simplest of model systems.

2 MORPHOLOGY OF BIOMEMBRANE FUSION

Studies using modern morphological and electrophysiological techniques have provided invaluable information on the process of biomembrane fusion. These techniques, which can be applied to intact biological systems, offer the spatial and temporal resolution so crucial in the analysis of a rapid and local process such as membrane fusion. Biomembrane fusion is consistently observed as a *local point* event that involves only a very small surface area of the interacting membranes. The initial fusion pore has a diameter of only 1 to 3 nm. Obviously, only relatively few lipid molecules (hundred to a few thousand) are directly involved in the fusion process.

3 BIOMEMBRANE FUSION DISSECTED

It has proven extremely difficult to discriminate fusogenic factors directly involved in the induction of membrane fusion from secondary factors involved only in processes that precede or follow actual membrane coalescence.

3.1 Trigger

Studies on *regulated exocytosis* (i.e., in response to an extracellular stimulus) have revealed a potential role for intracellular free calcium (Ca_i^{2+}), ATP, phospholipases, and GTP in the regulation of membrane fusion. A rise in Ca_i^{2+} was long thought to be both necessary and sufficient for the induction of exocytotic membrane fusion. Recent studies, however, indicate that though a rise in Ca_i^{2+} triggers exocytosis in many cell types, calcium is not directly responsible for membrane destabilization and the induction of membrane fusion. In some secretory systems, membrane fusion can even be triggered in the complete absence of calcium. ATP is required for the movement of secretory granules to the plasma membrane. Therefore, sustained release depends on ATP. Like calcium, however, ATP is often not a prerequisite for the fusion reaction itself. Many secretory processes are accompanied by the activation of phospholipases, and substantial amounts of diacylglycerol or unsaturated fatty acids may form. In contrast to calcium and ATP, these lipid products may play a direct role in the induction of membrane fusion by destabilizing the interacting membranes; experimental proof supporting this notion is still scarce, however. Finally, there is more and more evidence stressing the role of GTP-binding proteins in the regulation of membrane fusion during regulated exocytosis and *intracellular membrane traffic.* Extensive studies of cell-free systems reconstituting intracellular membrane fusion events have identified a proteinaceous multisubunit fusion machine that appears to regulate membrane fusion at multiple points in the secretory and endocytotic pathway. The core component is the highly conserved protein NSF (*N*-ethylmaleimide-sensitive factor), and its dephosphorylation may drive membrane fusion. However, a direct involvement of GTP-binding proteins or the intracellular fusion machine in membrane destabilization and the induction of membrane fusion has not (yet) been demonstrated. So far, a direct role for a protein in the induction of biomembrane fusion has been demonstrated only in the case of virus–membrane fusion (see Section 5).

3.2 Adhesion

Biomembrane adhesion involves removal of steric barriers, specific recognition, and local close apposition. Cytoskeletal elements and other cytosolic proteins play an important role in the spatial organization of the exocytotic machinery and may coregulate membrane fusion by controlling close apposition of membranes (e.g., in conjunction with calcium). The specificity of biomembrane fusion is guaranteed only if specific receptors exist to control membrane interaction. Evidence for such receptors has been obtained by studying regulated exocytosis in fusion-defective protozoa and in adrenal chromaffin cells. In addition, a sperm surface protein has been identified that interacts specifically with a protein from the zona pellucida of the egg cell. Finally, specific receptors for the NSF-containing intracellular fusion machine have been isolated from bovine brain. Similar proteins are found in other tissues and may have the same function, imposing specificity on an otherwise universal intracellular fusion machine. During membrane fusion, the lipid bilayers of the interacting membranes come into very close contact before they coalesce. Studies on pure lipid bilayers show that at a distance shorter than 30 A two forces dominate: a repulsive hydration force and an attractive hydrophobic force. The repulsive force arises from water tightly bound to the lipid head groups; the attractive force results from hydrophobic attraction between the hydrocarbon interiors of the membranes. Thus, model membrane fusion can be enhanced by incorporating lipids with a low head group hydration, reducing the repulsive hydration force, or by invoking defects in membrane lipid packing, which will expose more of the membrane interior and thereby increase the hydrophobic attraction. Similar principles may underlie the regulation of biomembrane fusion.

3.3 Fusion

During membrane fusion the equilibrium bilayer configuration of the membrane is abandoned temporarily (Figure 1a–c). Different molecular models have been proposed for the intermediate or "semi-fusion" stage of membrane fusion, the stage at which the outer lipid monolayers of the two membranes are continuous but the inner lipid monolayers are still separated (b1–b3 in Figure 1). A mechanism has been proposed in which two membranes first

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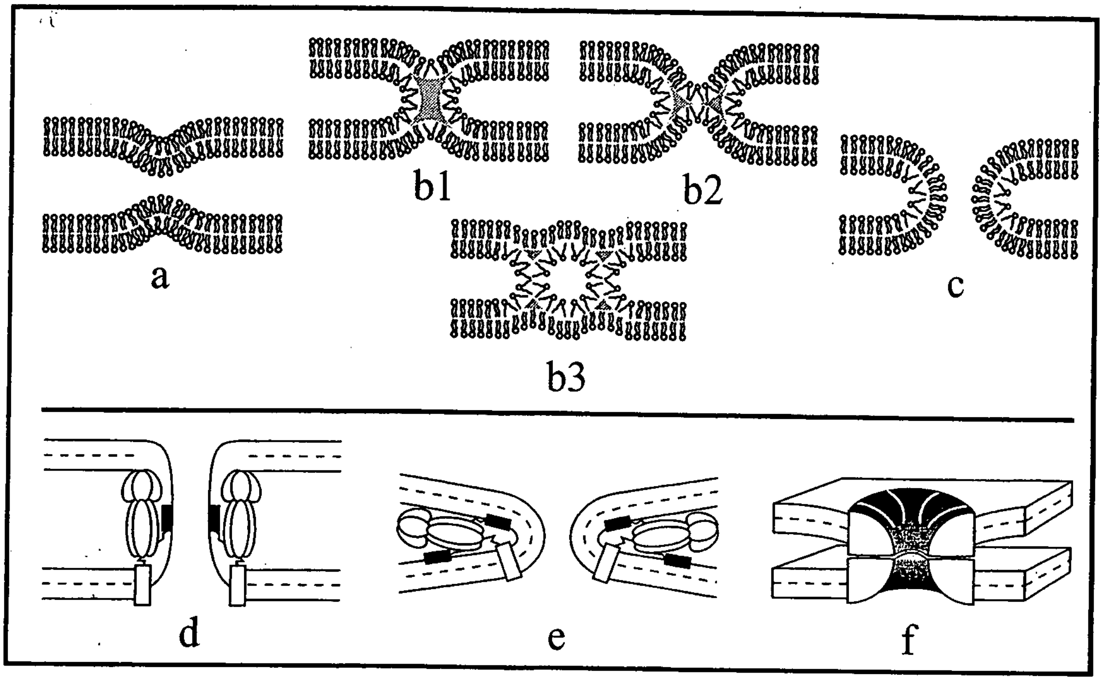

Figure 1. Molecular models for membrane fusion. (a=nc) Fusion of pure lipid membranes: (a) adhesion, (b) semifusion, (c) pore formation. Semifusion may involve a bilayer stalk (b1) and trans monolayer contact (b2), or an inverted lipid micelle (b3); hydrophobic interstices are shaded. (d=nf) Models of biomembrane fusion. Fusion pores formed after influenza virus=nmembrane fusion (d, e: viral membrane at the bottom, target membrane at the top); at low pH, HA remains in an upright position and the fusion peptides are extruded sideways (d), or HA tilts and the fusion peptides penetrate target and viral membrane (e). The fusion peptides form amphipathic α-helices (solid rectangles in d and e): the bulky apolar amino acids are present on one side, and the acidic amino acids on the opposite side of the α-helix. A model of the exocytotic fusion pore appears in f. For more details, see text.

join to form a so-called monolayer stalk (b1 in Figure 1). The stalk develops into a trans monolayer contact (b2 in Figure 1), which ruptures, resulting in a small aqueous pore (Figure 1c). A second model predicts the formation of an inverted lipid micelle at the semifusion stage (b3 in Figure 1). These models are mainly based on model membrane studies and on theoretical considerations (energy calculations); the actual involvement of the proposed semifusion intermediates in the act of (bio)membrane fusion has not been directly shown. The possibility that the real semifusion intermediate has yet another geometry, or is simply a local disordering of lipid molecules, cannot yet be excluded. Fusion is not completed before a pore has formed, connecting the two originally separated aqueous compartments. The initial pore probably forms spontaneously, but widening of the pore and release of contents may require influx of water (swelling) or ions.

4 SPECIFIC LIPID INVOLVEMENT IN BIOMEMBRANE FUSION

A number of "fusogenic" lipids have been proposed to be involved in the induction of membrane fusion. Some of these lipids, such as lysolecithin and monoacylglycerides, and acidic phospholipids, are not likely to be directly involved in the induction of membrane fusion. In contrast, inverted nonbilayer-preferring lipids ("type II lipids") are present in significant amounts in almost every biomembrane. An example is unsaturated phosphatidylethanolamine, present in all eukaryotic membranes. Type II lipids have a small head group relative to the cross-sectional area of their acyl chains. Upon isolation, they spontaneously form nonbilayer lipid structures with a molecular organization closely related to that of the putative inverted lipid micelle (b3 in Figure 1). Since membrane fusion involves a nonbilayer intermediate, a role for type II lipids in the regulation of biomembrane fusion is very appealing. Moreover, model membrane studies show that physiologically relevant factors such as diacylglycerol, unsaturated fatty acids, and hydrophobic proteins can induce membrane fusion by unmasking the phase preference of type II lipids. Similarly, factors involved in the regulation of biomembrane fusion may (in theory) directly initiate membrane fusion by locally inducing a bilayer-to-nonbilayer lipid structure transition. It should be noted that despite the probable role of type II lipids in biomembrane fusion, the molecular nature of the semifusion intermediate remains controversial. The fusogenic properties of type II lipids can be explained using any of the mentioned models for local point membrane fusion.

5 MOLECULAR MODELS OF BIOMEMBRANE FUSION

Current data indicate that proteins are directly involved in the induction of biomembrane fusion. The responsible *fusogenic* proteins, however, have been identified only in the case of the fusion of enveloped animal viruses with their target membrane. The best-characterized viral fusogenic protein is hemagglutinin (HA), a transmembrane glycoprotein of influenza virus. After being taken up by its host cell via endocytosis, influenza virus fuses with the

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endosomal membrane. The viral nucleocapsid is released into the cytoplasm and the virus starts to multiply. Membrane fusion is triggered by the low pH of the endosomal compartment, and HA is solely responsible for the low pH-induced fusion activity of influenza virus. The HA gene has been cloned, and the molecular structure of the ectodomain of HA has been determined by X-ray crystallography with 0.3 nm resolution. HA is present as a trimeric complex of identical monomers protruding some 13.5 nm from the viral membrane. Each monomer consists of two subunits, HA-1 and HA-2, linked by a disulfide bridge. HA-2 carries at its amino terminus a highly conserved and extremely hydrophobic stretch of about 20 amino acids, the so-called fusion peptide. The fusion peptide plays a crucial role in the induction of membrane fusion, and almost any change in its amino acid sequence by site-directed mutagenesis drastically influences the fusion activity of HA. At neutral pH, the three fusion peptides, one per monomer, are buried in the trimeric ectodomain of HA about 3 nm away from the viral membrane. At low endosomal pH, the interactions between the monomers in the HA trimer are partially lost and the fusion peptides are exposed. In addition, several HA trimers aggregate into a fusion complex. Membrane fusion occurs in the center of the fusion complex and is induced by the fusion peptides (solid rectangles in Figure 1d, e), which dehydrate the intermembrane space or create local defects in lipid packing of the interacting membranes, inducing them to fuse. The exact mechanism of HA-mediated membrane fusion is still under debate: it is not known whether the HA glycoproteins stay in an upright position (Figure 1d) or tilt to allow closer membrane contact (Figure 1e), nor has the molecular nature of the semifusion intermediate been settled yet.

Crucial to both models of HA-mediated membrane fusion is the regulated exposure of a hydrophobic entity, the fusion peptide, in the center of an oligomeric proteinaceous fusion complex. Indeed, this may turn out to be common to many biomembrane fusion processes. The fusogenic hydrophobic entity could be a terminal or an internal stretch of hydrophobic amino acids; alternatively, it could be a hydrophobic element in the tertiary structure, or a multiacylated part of a protein. For example, a membrane protein present in the head of mouse sperm (PH-30) has an internal stretch of amino acids with characteristics typical of viral fusion peptides, and PH-30 could be responsible for sperm–egg fusion. HA-Mediated membrane fusion may even prove to be a valuable model for regulated exocytosis. Morphological data obtained on the protozoan *Paramecium* also suggest the involvement of a multisubunit protein complex in exocytotic membrane fusion, and similar conclusions have been reached on exocytotic membrane fusion in mammalian cells based on electrophysiological measurements. Electrophysiological data indicate that the exocytotic fusion pore has the characteristics of an ion channel, and a model was proposed in which fusion is mediated by a multisubunit (gap junction-like) protein pore that spans both membranes (Figure 1f); the pore expands by incorporating lipids and by lateral dissociation of the pore subunits. However, electrophysiological studies of membrane fusion mediated by influenza HA also indicate the formation of a fusion pore that is very small and has characteristics very similar to those of exocytotic fusion pores. Therefore, in analogy to HA-mediated membrane fusion, exocytotic membrane fusion could involve a fusogenic protein on the vesicle membrane capable of destabilizing the cytoplasmic leaflet of the plasma membrane; and a mixed protein–lipid pore may form via a mechanism very similar to that described for HA-mediated membrane fusion (in Figure 1, compare d and e with f).

6 CONCLUSIONS AND PROSPECTS

The willingness of a biomembrane to fuse may be largely determined by a delicate equilibrium between bilayer- and nonbilayer-preferring lipids present in the membrane. During membrane fusion this equilibrium is locally disturbed, leading to a transient loss of the bilayer configuration of the membrane followed by membrane fusion. Proteins play a key role in this process, either by acting themselves as a fusogen or by locally producing a fusogen. Fusogenic proteins have been identified only in virus–membrane fusion, and we are just beginning to understand their mode of action. Well-synchronized biological systems and techniques that offer high temporal and spatial resolution will be required to identify the fusogenic proteins in other biomembrane fusion processes. The vast number of different biomembrane fusion processes may not all operate via the same fusion mechanism. An alternative mechanism may make use of lipid enzymes and a local change in lipid composition or transbilayer lipid asymmetry to induce membrane fusion. The determination of the relative importance of fusogenic proteins and membrane lipids in the regulation of biomembrane fusion will be a major challenge in future research.

See also CELL-CELL INTERACTIONS; CYTOSKELETON-PLASMA MEMBRANE INTERACTIONS; LIPIDS, STRUCTURE AND BIOCHEMISTRY OF.

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MEMORY AND LEARNING, MOLECULAR BASIS OF

Timothy E. Kennedy and Eric R. Kandel

Key Words

Afferent Describing a neural cell that conducts activity toward the central nervous system. The converse mode of conduction (i.e., toward the periphery) is called **efferent.**

Associative Learning The formation of a learned association between two stimuli or between a stimulus and a response. During **classical conditioning,** perhaps the best-studied form of associative learning, an initially neutral stimulus is associated with a highly effective stimulus by being repeatedly

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paired with it. Through this learned association, the previously neutral stimulus comes to evoke a learned response from the organism. In **operant conditioning,** another form of associative learning, the organism learns to produce a behavioral response in order to receive reinforcement. Unlike classical conditioning, the operant paradigm focuses on behavioral action and the consequences of that action. Much of motivated behavior can be described in these terms.

Channel Channels are transmembrane proteins that form pores in cell membranes, making them permeable to ionic species generating ionic currents. An electrical potential is present across all cell membranes. Channels may be sensitive to the electrical potential across the membrane and may be gated, opened or closed, by changes in membrane potential. Alternatively, ligand-gated channels are usually insensitive to membrane voltage and are gated by binding of a ligand such as a neurotransmitter. In some cases, such as the receptor for NMDA (*N*-methyl-D-aspartate), the channel is both ligand and electrically gated.

Classical Conditioning See **Associative Learning.**

Hippocampus A component of the limbic system implicated in the neural mechanisms of memory.

Neuron A nerve cell. Neurons send and receive information to and from other cells via synaptic connections.

Nonassociative Learning Learning in which the subject acquires information about the properties of a single stimulus. Thus the stimulus need not be associated with another stimulus or with a response. *Sensitization,* a form of nonassociative learning, describes a form of reflex enhancement due to the exposure of the organism to a stimulus, often a noxious stimulus. Conversely, *habituation* is a form of nonassociative reflex decrement due to the repeated presentation of an innocuous stimulus.

Operant Conditioning See **Associative Learning.**

Postsynaptic See **Synapse.**

Presynaptic See **Synapse.**

Synapse A specialized apposition between one neuron and another or between a neuron and an effector cell for the transmission or reception of an intercellular signal via a chemical neurotransmitter. The **presynaptic** side of the synapse initiates the signaling event. The **postsynaptic** side receives the signal. The consequences of synaptic activity can be excitatory or inhibitory for the postsynaptic cell. A presynaptic cell can influence the activity of a postsynaptic cell by opening or closing membrane channels and altering the postsynaptic membrane potential. Alternatively, presynaptic action may be neuromodulatory, activating second-messenger systems within the postsynaptic cell.

Learning is the process by which we acquire information about the environment. Memory is the retention of that information over time. An important goal of molecular neurobiology is the elucidation of the molecular mechanisms underlying learning and memory. To characterize these processes, it is necessary to identify the cellular sites within the nervous system responsible for learning and to specify mechanisms that mediate these changes. Significant progress in this regard has come from using invertebrates with relatively simple nervous systems, and from reduced vertebrate preparations such as the hippocampal slice. General principles of the molecular biology of learning and memory are beginning to emerge.

1 INSIGHTS FROM STUDIES OF INVERTEBRATES

1.1 SHORT- AND LONG-TERM SENSITIZATION IN *APLYSIA*

The nervous system of the marine mollusk *Aplysia* is made up of approximately 20,000 neurons. With this simple nervous system, *Aplysia* expresses a behavioral repertoire that includes a number of fundamental forms of learning: nonassociative learning such as sensitization and habituation, and associative learning such as classical and operant conditioning.

Sensitization of a reflex, a nonassociative form of learning, involves the enhancement of the strength of that reflex following the presentation of a noxious stimulus. In *Aplysia,* the gill- and siphon-withdrawal reflexes exhibit both short-term and long-term forms of sensitization. A touch to the siphon, part of *Aplysia's* delicate respiratory apparatus, evokes gill and siphon withdrawal, simple defensive withdrawal reflexes. Such stimulation activates siphon sensory neurons that synapse on interneurons and gill and siphon motor neurons (see Figure 1A). Noxious stimulation of the tail, such as a mild electric shock, activates facilitatory interneurons and produces behavioral sensitization, namely, an increase in the strength and duration of gill- and siphon-withdrawal following a touch to the siphon. The facilitatory interneurons consist of several types. Those best-studied use serotonin as a transmitter. Serotonin strengthens the synaptic connection between the siphon sensory neurons and the gill and siphon motor neurons, a process called presynaptic facilitation. A single shock to the tail produces short-term sensitization, behavioral reflex enhancement lasting seconds to minutes. Figure 1B illustrates a model of the biochemical events underlying short-term sensitization.

Like some forms of learning in vertebrates, repetition of training increases the duration of memory. With repeated stimulation, memory for short-term sensitization grades into memory for long-term sensitization, an enhancement of reflex strength lasting days to weeks. Unlike short-term sensitization, memory for long-term sensitization is dependent on the synthesis of RNA and protein. Inhibitors of protein and RNA synthesis applied during behavioral training, or during serotonin application, block the induction of the long-term process. In addition, long-term sensitization involves the growth of new synapses between the sensory neurons and motor neurons of the reflex. This increase in synaptic connectivity may represent the cellular mechanism underlying the long-term persistence of behavioral reflex enhancement.

As illustrated in Figure 1B, short-term sensitization involves the activation of cytoplasmic second-messenger systems and the covalent modification of existing cellular proteins. In contrast, the dependence on RNA and protein synthesis, and the induction of synaptic growth, suggest that long-term sensitization, in addition to activating cytoplasmic second-messenger systems, is dependent on the activation of a program of changes in gene expression via the activation of nuclear third messengers. Consistent with this idea, the injection into sensory neurons of an oligonucleotide containing the cAMP-responsive element, the binding site for the cAMP-responsive element binding protein transcription factor,

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Localization on the viral genome and nucleotide sequence of the gene coding for the two major polypeptides of the hepatitis B surface antigen (HBs Ag).

Charnay P, Mandart E, Hampe A, Fitoussi F, Tiollais P, Galibert F.

The structural gene coding for both polypeptides I and II which are the two major polypeptides of the Hepatitis B surface antigen, is found to be localized on the viral genome. This gene, referred to as gene S, is located in the partially single stranded region. It maps between positions 73.6 and 95.1% of the genome length. It is composed of 678 nucleotides, which correspond to a theoretical polypeptide of 25,422 molecular weight.

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Structural analysis of the gene coding for hepatitis B virus surface antigen and its product. [J Gen Virol. 1985]

Virion DNA of ground squirrel hepatitis virus: structural analysis and molecular cloning. [J Virol. 1982]

DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). [J Gen Virol. 1982]

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Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen.

Valenzuela P, Gray P, Quiroga M, Zaldivar J, Goodman HM, Rutter WJ.

DNA extracted from hepatitis B virus Dane particles has been cloned in bacteria using a plasmid vector. A full-length clone has been examined by restriction endonuclease analysis, and the nucleotide sequence of an 892-base pair fragment from cloned hepatitis B viral DNA encoding the surface antigen gene is reported. The amino acid sequence deduced from the DNA indicates that the surface antigens is a protein consisting of 226 amino acids and with a molecular weight of 25,398. The portion of the gene coding for this protein apparently contains no intervening sequences.

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Localization and nucleotide sequence of the gene coding for the woodchuck hepatitis virus surface antigen: comparison with the gene coding for the human hepatitis B virus surface antigen. [Proc Natl Acad Sci U S A. 1981]

Complete nucleotide sequence of hepatitis B virus DNA of subtype adr and its conserved gene organization. [Gene. 1984]

The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw. [Nucleic Acids Res. 1983]

Overlapping gene mutations of hepatitis B virus in a chronic hepatitis B patient with hepatitis B surface antigen loss during lamivudine therapy. [J Korean Med Sci. 2005]

Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. [Nucleic Acids Res. 1983]

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Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli.

Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P.

The complete nucleotide sequence of hepatitis B virus genome (subtype ayw) cloned in Escherichia coli has been determined using the Maxam and Gilbert method and the dideoxynucleotide method. This sequence is 3,182 nucleotides long. Location of the nonsense codons shows that the coding capacity of the L chain is larger than the coding capacity of the S chain. Eight open regions, able to code for polypeptide chains larger than 100 amino acids, have been located. Region 6, which is the largest, covers more than 80% of the genome. The gene S which codes for polypeptide I of the Hbs Ag and was previously located between coordinates 95.1 and 73.6 is contained in region 7.

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Nucleotide sequence of a cloned woodchuck hepatitis virus genome: comparison with the hepatitis B virus sequence. [J Virol. 1982]

The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw. [Nucleic Acids Res. 1983]

The complete nucleotide sequence of the cloned DNA of hepatitis B virus subtype adr in pADR-1. [Sci Sin [B]. 1987]

Hepatitis B virus genes and their expression in E. coli. [Nature. 1979]

Variant of hepatitis B virus isolated in Zimbabwe. [J Med Virol. 1994]

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Hepatitis B virus genes and their expression in E. coli.

Pasek M, Goto T, Gilbert W, Zink B, Schaller H, MacKay P, Leadbetter G, Murray K.

A composite DNA sequence of regions of hepatitis B virus, determined from a series of recombinant plasmids, reveals the genes for the surface antigen and the core antigen of the virus. The sequence of the core antigen shows it to be a DNA binding protein. The core antigen gene is expressed in Escherichia coli and when injected into rabbits the bacterial product induces antibodies which react with core antigen isolated from human sources.

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Expression of the hepatitis B virus surface, core and E antigen genes by stable rat and mouse cell lines. [J Mol Biol. 1982]

Core and E antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than genome length viral messenger RNAs. [J Mol Biol. 1983]

[Cloning and expression of the gene coding the hepatitis B virus surface antigen (HBsAg) in Escherichia coli] [Dokl Akad Nauk SSSR. 1983]

Two mammalian cell systems for propagation of the hepatitis B virus genome in extrachromosomal and chromosomally integrated states: production of the surface and e antigens. [Gene. 1985]

Cell surface expression of hepatitis B surface and core antigens in transfected rat fibroblast cell lines. [Gastroenterology. 1990]

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1: J Virol. 1983 Jun;46(3):920-36.

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Complete sequence of the Rous sarcoma virus env gene: identification of structural and functional regions of its product.

Hunter E, Hill E, Hardwick M, Bhown A, Schwartz DE, Tizard R.

The amino-terminal amino acid sequences of gp85 and gp37, the envelope glycoproteins of Rous sarcoma virus (RSV), were determined. Alignment of these sequences with the amino acid sequence predicted from the complete nucleotide sequence of the Prague strain of RSV, subgroup C (PR-C), has allowed us to delineate the env gene-coding region of this virus. The coding sequences for gp85 and gp37 have been placed in an open reading frame that extends from nucleotide 5045 to nucleotide 6862 and predict sizes of 341 amino acids (36,962 molecular weight) for gp85 and 198 amino acids (21,566 molecular weight) for gp37. Carbohydrate makes a significant contribution to the observed molecular weights of these polypeptides--the amino acid sequence contains 14 potential glycosylation sites (Asn-X-Ser/Thr) in gp85 and two in gp37. Experiments aimed at estimating the number of carbohydrate side chains yielded results consistent with most or all of these sites being occupied. Although an initiation codon is located early (codon 4) in the open reading frame, it is likely that splicing yields an mRNA on which translation initiates at the same AUG as that of the gag gene to produce a nascent polypeptide in which gp85 is preceded by a 62-amino-acid-long leader peptide. This leader contains the hydrophobic sequence (signal sequence) necessary for translocation across the endoplasmic reticulum and is completely removed from the env gene product during translation. The polyprotein precursor, Pr95env, is cleaved to gp85 and gp37 at the carboxyl side of the basic sequence:-Arg-Arg-Lys-Arg-. gp85 is attached through a disulphide linkage to gp37, and although the positions of the cysteines involved in this linkage are not known, the presence of a 27-amino-acid-long hydrophobic region at the carboxy-terminus of gp37 is consistent with its role as a membrane anchor for the viral glycoprotein complex. The location of host range variable regions with respect to the possible tertiary structure of the complex is discussed.

PMID: 6304351 [PubMed - indexed for MEDLINE]

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Avian sarcoma virus gag and env gene structural protein precursors contain a common amino-terminal sequence. [Proc Natl Acad Sci U S A. 1984]

Amino-terminal deletion mutants of the Rous sarcoma virus glycoprotein do not block signal peptide cleavage. [J Cell Biol. 1986]

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intracellular transport. [J Cell Biol. 1986]

DNA sequence of the viral and cellular src gene of chickens. 1. Complete nucleotide sequence of an EcoRI fragment of recovered avian sarcoma virus which codes for gp37 and pp60src. [J Virol. 1982]

A charged amino acid substitution within the transmembrane anchor of the Rous sarcoma virus envelope glycoprotein affects surface expression but not intracellular transport. [J Cell Biol. 1987]

Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. [J Virol. 1987]

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1: Science. 1984 Jul 27;225(4660):421-4.

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Sequence of the envelope glycoprotein gene of type II human T lymphotropic virus.

Sodroski J, Patarca R, Perkins D, Briggs D, Lee TH, Essex M, Coligan J, Wong-Staal F, Gallo RC, Haseltine WA.

The sequence of the envelope glycoprotein gene of type II human T lymphotropic virus (HTLV) is presented. The predicted amino acid sequence is similar to that of the corresponding protein of HTLV type I, in that the proteins share the same amino acids at 336 of 488 residues, and 68 of the 152 differences are of a conservative nature. The overall structural similarity of these proteins provides an explanation for the antigenic cross-reactivity observed among diverse members of the HTLV retrovirus family by procedures that assay for the viral envelope glycoprotein, for example, membrane immunofluorescence.

PMID: 6204380 [PubMed - indexed for MEDLINE]

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Serological cross-reactivity between envelope gene products of type I and type II human T-cell leukemia viruses. [Proc Natl Acad Sci U S A. 1984]

Delineation of type-specific regions on the envelope glycoproteins of human T cell leukemia viruses. [J Immunol. 1991]

Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. [Science. 1985]

Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. [Cell. 1986]

Detection of antibodies to human T-lymphotropic virus type III by using a synthetic peptide of 21 amino acid residues corresponding to a highly antigenic segment of gp41 envelope protein. [Proc Natl Acad Sci U S A. 1986]

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Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions.

Modrow S, Hahn BH, Shaw GM, Gallo RC, Wong-Staal F, Wolf H.

Independent isolates of human immunodeficiency virus (HIV) exhibit a striking genomic diversity, most of which is located in the viral envelope gene. Since this property of the HIV group of viruses may play an important role in the pathobiology of the virus, we analyzed the predicted amino acid sequences of the envelope proteins of seven different HIV strains, three of which represent sequential isolates from a single patient. By using a computer program that predicts the secondary protein structure and superimposes values for hydrophilicity, surface probability, and flexibility, we identified several potential antigenic epitopes in the envelope proteins of the seven different viruses. Interestingly, the majority of the predicted epitopes in the exterior envelope protein (gp120) were found in regions of high sequence variability which are interspersed with highly conserved regions among the independent viral isolates. A comparison of the sequential viral isolates revealed that changes concerning the secondary structure of the protein occurred only in regions which were predicted to be antigenic, predominantly in highly variable regions. The membrane-associated protein gp41 contains no highly variable regions; about 80% of the amino acids were found to be conserved, and only one hydrophilic area was identified as likely to be accessible to antibody recognition. These findings give insight into the secondary and possible tertiary structure of variant HIV envelope proteins and should facilitate experimental approaches directed toward the identification and fine mapping of HIV envelope proteins.

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Sequence divergence and open regions of RNA secondary structures in the envelope regions of the 17 human immunodeficiency virus isolates. [Nucleic Acids Res. 1989]

Computer predictions of antigenic domains in human immunodeficiency virus-1 envelope glycoprotein: comparison with reported experimental da[Virus Genes. 1990]

Helper T-cell antigenic site identification in the acquired immunodeficiency syndrome virus gp120 envelope protein and induction of immunity in mice to the native protein using a 16-resid[Proc Natl Acad Sci U S A. 1987]

Characterization of human antibody-binding sites on the external envelope of human immunodeficiency v[J Gen Virol. 1991]

Conserved and exposed epitopes on intact, native, primary human immunodeficiency virus type 1 virions of group M[J Virol. 2000]

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MECHANISMS OF VIRAL MEMBRANE FUSION AND ITS INHIBITION

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Key Words viral entry, influenza hemagglutinin, HIV-1 gp41, trimer-of-hairpins, spring loaded, prehairpin intermediate

■ **Abstract** Viral envelope glycoproteins promote viral infection by mediating the fusion of the viral membrane with the host-cell membrane. Structural and biochemical studies of two viral glycoproteins, influenza hemagglutinin and HIV-1 envelope protein, have led to a common model for viral entry. The fusion mechanism involves a transient conformational species that can be targeted by therapeutic strategies. This mechanism of infectivity is likely utilized by a wide variety of enveloped viruses for which similar therapeutic interventions should be possible.

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INTRODUCTION

The fusion of lipid bilayers is central to a number of diverse biological processes, such as fertilization, vesicle trafficking, muscle development, and viral infection. To date, the most extensively studied of these events is the membrane fusion of enveloped viruses. When an enveloped virus infects a host cell, its membrane fuses with the host-cell membrane, allowing the contents of the virus to be transferred to the host. This fusion event is mediated by virally encoded surface glycoproteins.

Scope of this Review

In this review we focus on the fusion mechanisms mediated by two viral proteins, influenza hemagglutinin (HA) and human immunodeficiency virus type-1 (HIV-1) envelope protein (Env). We present these two viral proteins together, yielding a more complete picture of viral membrane fusion than can be accomplished by focusing on either individually. Biochemical and structural studies on the fusogenic proteins are emphasized. In addition, we discuss the current status of HIV-1 membrane fusion inhibitors.

Other Viral Membrane Fusion Reviews

Clearly, there are many areas of study in the field of viral membrane fusion. Although we try to at least touch on most subjects, many more thorough reviews on specific topics exist. Influenza HA has long been the paradigm for viral-mediated membrane fusion and has an extensive history in the literature. In 1987, an early, informative review was provided by Wiley & Skehel (1). Since then, a number of comprehensive reviews have appeared (2–10). The HA-mediated merging of lipid bilayers has been reviewed (11, 12), as has the role of fusion peptides in membrane fusion (13, 14). Recently, HIV-1 Env was reviewed (9, 15–17). The HIV-1 coreceptor has been thoroughly discussed (18, 19), and the roles of coiled coils in viral membrane fusion and vesicle membrane fusion have been compared (20, 21). Thorough reviews of inhibiting HIV-1 viral entry have been provided (22–25), and finally, the fusion events of other viral families have been covered (7, 26).

ARCHITECTURE OF VIRAL MEMBRANE FUSION PROTEINS

Enveloped animal viruses encompass many viral families, each with a distinct virion morphology, genome structure, and life cycle (27). Despite extensive differences, these viruses also share common features. For example, most enveloped viruses bud from host-cell membranes, thereby acquiring a cell-derived lipid bilayer. Some viruses, such as paramyxoviruses, bud from the plasma membrane whereas other viruses, such as flaviviruses, bud from internal membranes like the endoplasmic reticulum. Enveloped viruses also share a common mechanism of infection. A glycoprotein on the surface of the virus mediates fusion between the viral membrane and the host-cell membrane, allowing release of the viral contents into the host cell.

The fusion glycoproteins of enveloped viruses, typically type-I integral membrane proteins, are encoded by the virus and synthesized by the infected cell. They are incorporated into the host-cell membrane and subsequently into the budding virus. Fusion glycoproteins comprise (*a*) a cleavable amino-terminal signal sequence directing it to the endoplasmic reticulum, (*b*) a large extraviral region (also referred to as the ectodomain), (*c*) a stop-transfer region that forms a transmembrane helix to anchor the protein in the viral membrane, and (*d*) a cytoplasmic tail. The cytoplasmic tail ranges from 20 amino acids for paramyxoviruses to ~150 amino acids for some retroviruses. Typically, the envelope glycoproteins are synthesized as a precursor and then cleaved into two subunits that remain closely associated with each other. These proteins form higher-order oligomers and are glycosylated. Because of their location on the outer surface of the viral membrane, much of the host's immune response targets these glycoproteins. Also, these envelope glycoproteins contain a short region within their sequence called the "fusion peptide," which is required for mediating membrane fusion (for reviews, see 13, 14). The fusion-peptide region, rich in hydrophobic and glycine residues, interacts with the host-cell membrane at an early stage of the membrane-fusion process.

Viral entry is initiated when the surface glycoprotein binds to the appropriate cellular receptor(s) on the host-cell surface. Subsequent to binding, some enveloped viruses fuse with the cell-surface membrane at neutral pH, whereas others are endocytosed into clathrin-coated pits and fuse with the endosomal membrane when the pH is lowered. Viruses such as HIV-1 and human respiratory syncytial virus use the former method of viral entry, whereas viruses such as influenza and rabies utilize the latter.

Viral envelope fusion proteins proceed through a series of conformational changes in order to mediate fusion with the host cell. The initial cleavage of the glycoprotein precursor leaves the complex in a metastable state, primed for fusion, although the fusion peptide is not exposed. Conformational changes occur in response to binding to the host-cell receptors or exposure to low pH, exposing the fusion peptide and allowing juxtaposition of the viral and host-cell membranes, which leads to membrane fusion.

MODEL STUDIES OF VIRAL MEMBRANE FUSION: Influenza HA and HIV-1 Env

Several decades of research illuminate a complex and elegant process by which influenza HA mediates viral membrane fusion, making it the prototypic enveloped viral membrane fusion glycoprotein (for reviews, see 1, 2, 7). More recently, there is increasing emphasis on biochemical and structural studies of the envelope glycoprotein of HIV-1. Although these two viruses are members of distinct viral families differing in genome organization, physical morphology, and replication strategies, at the molecular level they utilize a similar mechanism for gaining access to the interior of a host cell. Combining the experimental information available for these distinct virus glycoproteins creates a more complete picture of the viral entry process than when either virus is considered individually.

HA and Env are the sole viral proteins required for membrane fusion with the host cell by influenza and HIV-1, respectively. They are both synthesized as fusion-incompetent precursors, termed HA0 (for HA) and gp160 (for Env). They are then proteolytically cleaved into two subunits, a surface subunit and a transmembrane subunit, activating the fusion potential of the glycoproteins. The surface subunit of influenza, HA1, remains covalently associated to the transmembrane subunit, HA2, through a disulfide bond (Figure 1*a*). The interaction between the cleaved products of gp160 (a surface subunit, gp120, and a transmembrane subunit, gp41) is noncovalent. The surface subunits are responsible for recognizing and binding to specific receptors on the host cell. The transmembrane subunits contain the fusion-peptide region at their amino terminus and are anchored in the viral membrane via hydrophobic membrane-spanning helices. Despite numerous similarities between the two glycoproteins, the sites of entry of the two viruses differ. After HA1 binds to its receptor, sialic acid, the influenza virus is endocytosed and fusion is initiated with the endosomal membrane at low pH. In the case of HIV-1, gp120 binds to a cellular receptor, CD4, and subsequently a coreceptor, one of a family of seven transmembrane helix chemokine receptors (18), and the entry event is accomplished at the cellular surface at neutral pH.

Conformational Changes on Fusion Activation

The realization 20 years ago that exposure to low pH activates the membrane fusion potential of HA (28–30) led to intense scrutiny of the effect of low pH on HA structure. Consequently, prior to high-resolution structural information, it was discerned that exposure to low pH induces irreversible conformational changes in HA. Similarly, biochemical studies indicate HIV-1 Env changes conformation in response to CD4 binding—its fusion-activation signal.

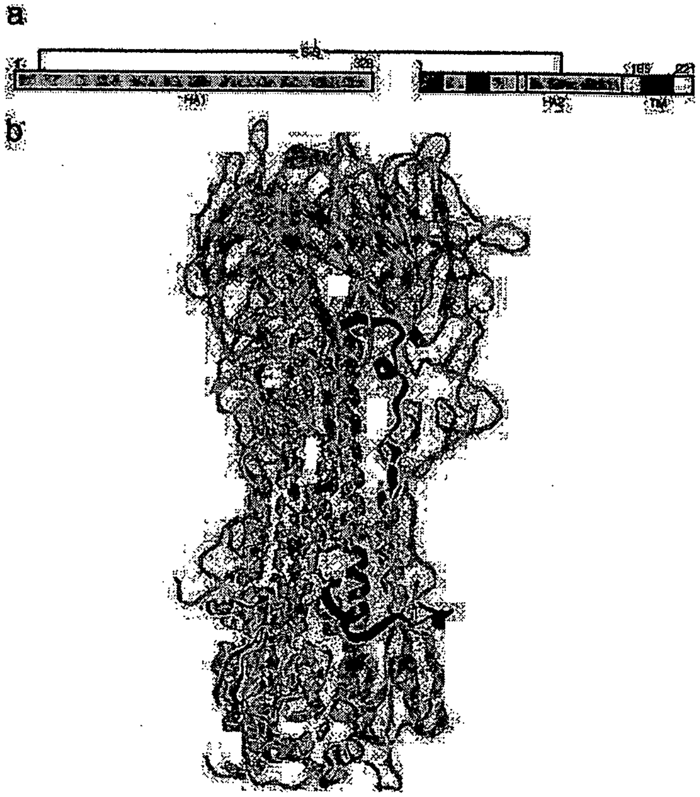


Figure 1 High-resolution structure of native influenza hemagglutinin (HA). (*a*) The primary structure of processed HA, including a disulfide bond between residue 14 of HA1 and residue 137 of HA2. (Color) The residues present in the bromelain-released form of processed HA (BHA) X-ray crystal structure in *b*. (*b*) The X-ray crystal structure of the trimeric BHA, including all of HA1 and the first 175 residues of HA2 (61). Three HA1 monomers (yellow) sit atop a stalk-region composed of three HA2 monomers (two gray, one mostly blue). The first 20 residues of the predicted fusion peptide (green) are indicated. Residues 55–76 (red), which are in a loop conformation, become helical during the HA-mediated fusion process (Figures 2 and 3). Residues 106–112 (orange), helical here, change to a loop conformation to form the trimer-of-hairpins during the fusion process (Figure 3). Figure drawn with Insight II 98.0 (Molecular Simulations, Inc, San Diego, CA).

First, on exposure to fusion-activating conditions (a temporary lowering of pH for HA and CD4 exposure for Env), both proteins become more hydrophobic: HA forms aggregates and can bind detergents and liposomes after exposure to low pH (31), whereas both proteins acquire the ability to bind bis-ANS, a hydrophobic fluorescent dye (32, 33). Second, HA and Env undergo changes in their proteolytic susceptibility (34, 35). Third, there is evidence for alteration of the interaction between the surface and transmembrane subunits. Electron microscopy identifies a significant change in shape of HA, including a partial dissociation of globular head domains on the top of the molecule (31, 36). In laboratory-adapted strains of HIV-1, CD4 exposure can cause the dissociation of gp120 from gp41, a process known as shedding (37). Fourth, mutagenesis studies suggest the existence of distinct conformations. Specific point mutations in predicted coiled-coil domains of the glycoproteins do not alter the proteolytic processing or surface expression, yet they jeopardize infectivity and glycoprotein-mediated membrane fusion. Most likely, these mutations disrupt the fusion-active state of the protein, but not the native, nonfusogenic form (38–45). And fifth, the epitope accessibility of the proteins is altered.

In the case of HA, the changes in antigenicity with a change in pH indicate that structural rearrangements occur throughout the molecule, as the recognition of most epitopes is altered when HA is exposed to low pH (46–49). For example, monoclonal antibodies reactive with the tip of native HA are unable to immunoprecipitate HA after low-pH–induced conformational changes (50). Likewise, antibodies specific for epitopes exposed in the low-pH–activated HA, such as antibodies raised against the amino-terminal fusion peptide, are unable to immunoprecipitate native HA (50, 51).

In HIV-1, gp120 undergoes changes in antigenicity early in the fusion process. For example, the V3 loop on gp120 is recognized after receptor binding (52), and epitopes on gp41 become more accessible (53). Also, following binding of CD4, novel epitopes are revealed on the surface of gp120 that allow recognition by neutralizing antibodies (54). Neutralizing antibodies, such as 17b, block binding of gp120/CD4 complexes to the coreceptor (55, 56), which suggests that CD4 binding exposes the previously hidden coreceptor binding site (57). Consistent with this chain of events, after exposure to soluble CD4 (58), HIV-1 can fuse with cells expressing a coreceptor and lacking CD4 (59).

Structure/Function Studies

After decades of work, structure/function studies on the glycoproteins of both influenza and HIV-1 provide structural details of the predicted conformational changes. The conformational changes of HA are well defined, with high-resolution X-ray crystal structures available for three states: unprocessed precursor (HA0), and proteolytically processed HA in both the native and low-pH–activated conformations. There are also high-resolution data for gp120 and gp41. Some of these structures, as well as other important structure/function studies, are described below.

High-Resolution Structure of Influenza HA in the Native Conformation In 1981, Wilson et al (61) provided the first high-resolution view of a viral envelope glycoprotein. The native structure of influenza HA was solved by crystallization of a large portion of the HA ectodomain that was cleaved from the viral surface by bromelain (BHA) (60, 61) (Figure 1*b*). BHA lacks the hydrophobic region that anchors HA to the viral membrane. It is a long trimer that extends 135 Å. At the top of the structure are three exposed globular head domains consisting strictly of HA1 and responsible for binding to sialic acid. The head domains sit atop a stalk, composed of the remainder of HA1 and HA2. This stalk contains a central trimeric coiled coil at its core, composed strictly of HA2. The amino terminus of HA2, which contains the fusion peptide, is buried in the native structure. The fusion peptide is located approximately 35 Å from the carboxy terminus of the structure and 100 Å from the distal tip.

Because BHA was prepared at neutral pH from virus that had not been exposed to low pH, it presumably represents the native conformation of the HA ectodomain (i.e. the conformation of HA on the surface of the virus following proteolytic processing yet preceding low-pH activation). Structural details are consistent with this interpretation. First, the distal end of the molecule (away from the presumed location of the viral-spanning region) is composed of HA1, and HA1 recognizes and binds to cell receptors. Second, the fusion peptide is sequestered in the interior of the structure, unavailable for mediating fusion. Earlier biochemical studies predicted the burial of the fusion peptide in the prefusogenic conformation.

The BHA structure was groundbreaking but left many questions unanswered. Most important, what conformational changes could expose the fusion peptide from its buried location in the folded structure, and how could the exposed fusion peptide interact with the cell membrane when they were over 100 Å apart?

The Spring-Loaded Model Twelve years after the native HA structure was solved, synthetic peptide experiments led to a structural model of low-pH–induced conformational changes in HA (62). Computational methods identified a region of HA2 (residues 54–81) with a high propensity for forming a coiled coil. However, in the native X-ray crystal structure, most of this region maintains an extended loop conformation (residues 55–75) (Figure 1*b*). Biophysical studies on the synthetic peptide corresponding to this region confirmed that, in fact, it forms a coiled coil in solution. Therefore, it was proposed that in the transition from the prefusogenic, native structure to the fusogenic, low-pH–activated structure, the conformation of these residues changes from a loop to a coiled coil. This structural rearrangement would extend the central trimeric coiled coil and propel the fusion peptide to the opposite end of the molecule, allowing it to interact with the host-cell membrane (Figure 2). This proposal is called the spring-loaded mechanism, emphasizing the idea that the prefusogenic structure is primed for the conformational change that leads to fusion (62). One year later, the X-ray crystal structure of low-pH–activated HA was solved and confirmed the structural features of the spring-loaded mechanism [a more recent structure is depicted in Figure 3*a*] (63). In this fusogenic structure, residues 55–75 form an extension of the central coiled coil of the native structure. Although the fusion peptide is not present in the structure, this conformational change is expected to move the fusion peptide 100 Å from its position

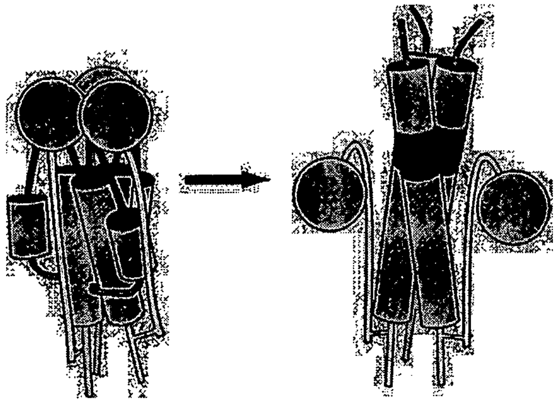


Figure 2 Spring-loaded mechanism for viral membrane fusion (62). In the native conformation of influenza hemagglutinin (HA) (*left*), the HA1 subunits (yellow balls) occupy the distal end of the structure, atop a trimeric coiled coil region of HA2 (blue). The fusion peptides (green) are buried in the core of the HA2 structure. On induction of low pH, fusion-activating conformational changes occur (*right*). The noncovalent interactions between HA1 and HA2 weaken and the loop regions of HA2 (red) "spring" into helical conformations, extending the central trimeric coiled coil and propelling the fusion peptides to the top of the structure to interact with the target membrane. (Adapted from Reference 62.)

in the native conformation. Mutations that reduce the helical propensity of the spring-loaded region reduce membrane fusion, indicating the importance of the loop-to-helix transition in the fusion event (39). With prolines at both positions 55 and 71, HA maintains surface expression and proteolytic maturation, yet no fusion activity occurs.

How is a shift in pH able to cause such major conformational changes? Evidence suggests the native prefusogenic conformation of HA is metastable, separated from the stable fusogenic state by a kinetic barrier (64). The pH change serves to destabilize the native state, making it easier to overcome the kinetic barrier. Indeed, low pH per se is not required for HA-mediated fusion. At neutral pH, high temperature or a chemical denaturant can activate the spring-loaded conformational change of HA and, therefore, HA-mediated membrane fusion (64).

High-Resolution Structures of Fusion-Active Conformations In order to obtain structural information on HIV-1 gp41, a recombinantly expressed gp41 ectodomain lacking the amino-terminal fusion peptide was subjected to proteolysis (65). This technique, termed protein dissection, potentially reveals stable subdomains of a protein or protein complex. After gp41 was treated with proteases, a trimeric helical subdomain of gp41 remained. The subdomain is composed of two discontinuous peptides, N51 and C43, and is extremely stable, only unfolding at extreme temperatures. Generally, peptides from these two regions of gp41 are called N-peptides and C-peptides because they originate from the amino-terminal and

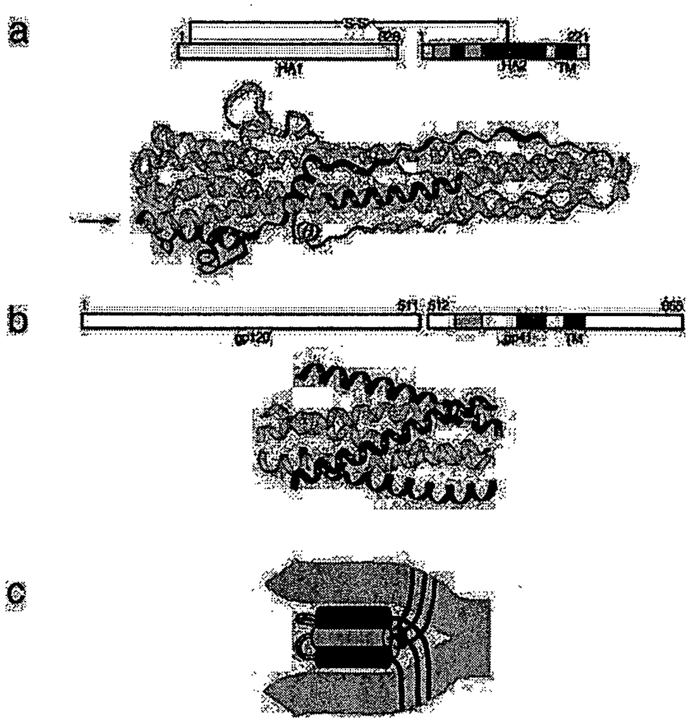


Figure 3 High-resolution views of fusion-active envelope glycoproteins and a model of the trimer-of-hairpins. (*a*) Primary structure and X-ray crystal structure of low-pH–activated influenza hemagglutinin (HA) (72; see also 63). The primary structure depicts processed HA, including a disulfide bond between residue 14 of HA1 and residue 137 of HA2. (*Color*) The residues present in the X-ray crystal structure. The X-ray crystal structure represents a stable trimeric domain of fusogenic HA2. One of the three helices (blue and red) that form the central coiled coil is surrounded by carboxy-terminal residues in an extended conformation (purple). Two monomers (gray) complete the trimer-of-hairpins. The amino- and carboxy-terminal ends of the protein are located at the same end of the structure. Residues 55–76 (red) participate in the loop-to-helix conformational change in the spring-loaded mechanism. Residues 106–111 (orange, arrow) change from helix-to-loop. (*b*) Primary structure and X-ray crystallography structure of fusogenic gp41 (67; see also 68, 69). The primary structure depicts processed gp120 and gp41. (Purple, blue) The residues present in the high-resolution structure. The X-ray structure is a trimer-of-hairpins, with three helical N-peptides in a coiled-coil conformation surrounded by three helical C-peptides. The amino- and carboxy-terminal ends of gp41 are located at the same end of the structure. (*c*) Model of the trimer-of-hairpins of HIV-1 that juxtaposes the viral and cellular membranes. (Blue) The N-peptides; (purple) the C-peptides. The fusion peptide (*green*) is inserted in the host cell membrane. The transmembrane domain of gp41 (*black*) is located in the viral membrane. High-resolution structures drawn with Insight II 98.0 (Molecular Simulations, Inc).

the carboxy-terminal regions of the gp41 ectodomain, respectively. Biophysical analysis led to the proposal that the N-peptides form a central trimeric coiled coil, with helical C-peptides bound to the outside of the coiled coil, packed antiparallel to the helices in the N-peptide coiled-coil core. The same protein dissection and biophysical analyses were performed on the ectodomain from simian immunodeficiency virus (SIV), a closely related retrovirus, and yielded the same results (66).

The structural model predicted from these biophysical studies was subsequently confirmed by X-ray crystallography for both HIV-1 (67–69) (Figure 3*b*) and SIV (70). The structure is described as a trimer-of-hairpins. In the trimer-of-hairpins, three helical C-peptides bind to the outside of the coiled-coil core of N-peptides in an antiparallel manner, with each C-peptide binding to a conserved hydrophobic groove formed by two N-peptides. Therefore, the intervening sequence of gp41 (not present in the structure) would be required to loop around from the base of the coiled coil to fold the C-peptide back to the same end of the molecule (Figure 3*c*). Indeed, a solution structure of SIV gp41 shows the loop between the two helical regions (71).

It is interesting that the low-pH–activated HA2 structure also contains a trimer-of-hairpins (Figure 3*a*) (63, 72). In addition to the loop-to-helix spring-loaded conformational change in influenza HA, there is a significant helix-to-loop change between native and low-pH–activated HA. Six carboxy-terminal residues near the base of the central trimeric coiled coil of the native structure become a loop in the low-pH–activated structure (residues 106–112) (see Figures 1 and 3*a*), reversing the direction of what was the carboxy-terminal end of the coiled coil. An additional low-pH–activated HA structure that was recently solved unequivocally demonstrates that the amino- and carboxy-terminal regions of the ectodomain come together at the same end of the folded structure, similar to HIV-1 (Figure 3) (72).

There are many reasons why the trimer-of-hairpins of HIV-1 gp41 and influenza HA are thought to represent the fusion-active conformation of the envelope glycoproteins. First, for HA, the molecules used for the structural investigations were prepared by exposure to low pH (63, 72), and low pH is required to activate the fusion potential of HA (28–30). Second, in the absence of the surface subunits, the transmembrane subunits are expected to fold to their fusogenic state. Indeed, in HIV-1, conformational changes induced by receptor binding and required for fusion activation are likely to ultimately lead to the shedding of gp120 (37). In influenza, biochemical studies predict a partial dissociation of HA1 from HA2 on fusion activation, and HA2 of influenza folds into its low-pH–activated form in the absence of HA1 (62, 73). Third, the final fusogenic structures of HA and HIV-1 described above are extremely stable and unlikely to unfold after they are formed (65, 72). In contrast, the native, metastable state of HA is easily altered by low pH, heat, or chemical denaturants (64). The native state of Env is also altered by increasing temperature (74). Fourth, the resulting conformational changes in the soluble ectodomain fragments of HA are consistent with biochemical and morphological observations of the conformational changes that are required for HA-mediated fusion (31, 35, 36, 46, 47, 49–51). As mentioned above, these observations predict an exposure of the fusion-peptide region. The low-pH–activated structures display significant conformational differences from the native structure, including a spring-loaded change that would presumably displace the fusion-peptide region from a buried region to an exposed location on the amino terminus of the trimeric coiled-coil core (63, 72). Also, the high-resolution structures are consistent with the shape of HA observed previously in electron micrographs of low-pH–activated HA on the viral surface (36). Fifth, specific mutations that alter fusion activity map to regions that change conformation between the native and low-pH–activated structures in influenza or to the central trimeric coiled coil of the HIV-1 gp41 structure (38–46, 63). These mutations do not affect processing or cell-surface expression of the membrane-fusion proteins and, thus, presumably do not substantially alter the native structure. Specifically, as mentioned earlier, proline substitutions in the spring-loaded region of HA ablate fusion (39). Finally, these structures are consistent with the biochemical data of inactivated virus. When influenza is exposed to low pH in the absence of a target membrane, it is inactivated, presumably by inserting its fusion peptide into its own or other viral membranes (75–77). In the low-pH–activated structures, the amino and carboxy termini are located at the same end of the molecule, just as they would be in the inactivated form. Indeed, a monoclonal antibody against residues 105–113 of HA2 (see Figure 3*a*) binds to the tip of HA distal to the viral membrane of inactivated virus (48). Therefore, the trimer-of-hairpins structure is thought to define the fusion-active conformations of these viral envelope glycoproteins that occur either concomitantly or just after membrane fusion. Hereafter, these structures are referred to as fusogenic.

gp41-Derived Inhibitory Peptides The conclusion that the trimer-of-hairpins is indeed the fusogenic state is consistent with the inhibitory activity of gp41-derived peptides. Several years before the structural studies on HIV-1 gp41, synthetic N- and C-peptides were shown to inhibit HIV-1 infection (65, 78–81). The C-peptides, effective at nanomolar concentrations, are much more potent than N-peptides, which require micromolar concentrations for effectiveness. It was proposed that C-peptides act by binding to or near the predicted helical region downstream from the fusion peptide, which corresponds to the N-peptide region (65, 81, 82), and therefore inhibits infection in a dominant-negative manner (65).

Several experimental observations support the dominant-negative inhibition hypothesis. First, the inhibitory activity of the C-peptide is substantially decreased in the presence of an equal amount of N-peptide (65). Second, mutant C-peptides that destabilize formation of the trimer-of-hairpins structure also demonstrate weakened antiviral potency (83, 84). Third, the hydrophobic binding surface on the N-peptide coiled-coil core to which the C-peptides bind is highly conserved between HIV-1 and SIV. Indeed, the comparable SIV C-peptide inhibits HIV-1–mediated membrane fusion (70). Fourth, viruses that develop resistance to

C-peptides contain substitutions of several residues in the N-peptide region (85). And fifth, epitope-tagged C-peptide can immunoprecipitate gp41 (86). Thus, there is compelling evidence that C-peptides inhibit formation of the trimer-of-hairpins in a dominant-negative manner by binding to the N-peptide region of gp41.

A conundrum remains, however, with a simple dominant-negative model (16). How can the C-peptides—in an intermolecular interaction that occurs at nanomolar concentrations—prevent formation of an intramolecular interaction in which the N- and C-peptide regions of the same gp41 molecule interact to form a hairpin?

A Transient Fusion Intermediate The conundrum is partially solved by the proposal of a transient intermediate in the fusion process—an intermediate formed after receptor binding but before formation of the trimer-of-hairpins (16, 69, 86) (Figure 4). In this intermediate, termed prehairpin, the N-peptide region is exposed, vulnerable to binding by synthetic C-peptides. To finish solving the conundrum, it is proposed that the C-peptide–inhibited gp41 ultimately becomes irreversibly inactivated.

Several experimental observations support the existence of the prehairpin intermediate. For example, C-peptides must be present during exposure to the host cell in order to be effective. If virus is preincubated with C-peptide, followed by removal of the C-peptide before addition of the target membrane, infection is not inhibited (86). Also, epitope-tagged C-peptide can immunoprecipitate gp41, but only after exposure to CD4 (and for some HIV-1 isolates, a coreceptor), demonstrating that a receptor-mediated conformational change is required to expose the N-peptide region (86).

The kinetics of C-peptide inhibition suggest that the transient intermediate experiences a lifetime of many minutes (16, 87). Conformational changes resulting from receptor binding begin in under 1 min (32), but C-peptides maintain potency even if added up to 15 min after receptor exposure (87).

A transient intermediate has also been proposed for influenza HA (2, 9), although it has yet to be verified experimentally. In influenza, it is likely that the conformational changes between the native structure and the fusogenic structure occur sequentially. First, the spring-loaded mechanism occurs, propelling the fusion peptide up to the target membrane; this step corresponds to the formation of the prehairpin in HIV-1. Next, the bottom of the trimeric coiled coil reverses direction to fold the molecule in half, bringing the two membranes into close contact, just as in the trimer-of-hairpins for HIV-1.

As mentioned above, N-peptides derived from HIV-1 gp41 exhibit anti-HIV activity, but with much lower potency than do the gp41 C-peptides (65, 79). The inhibition mechanism of N-peptides is unknown, however, because of their tendency to aggregate. It is possible N-peptides inhibit by targeting the C-peptide region of gp41 (65); alternatively, they may intercalate into the N-peptide region (44, 79). Recently, HIV-1 fusion inhibitors that specifically target the C-peptide region of gp41 were identified (88; DM Eckert & PS Kim, unpublished observations) (see also below), confirming the accessibility of the C-peptide region prior to

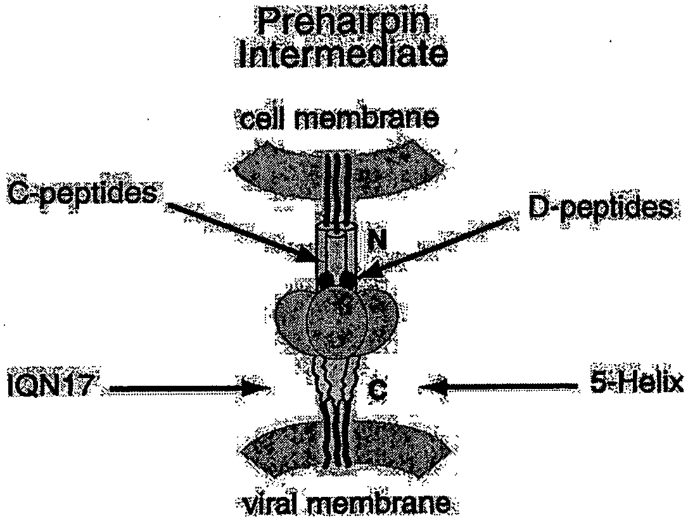


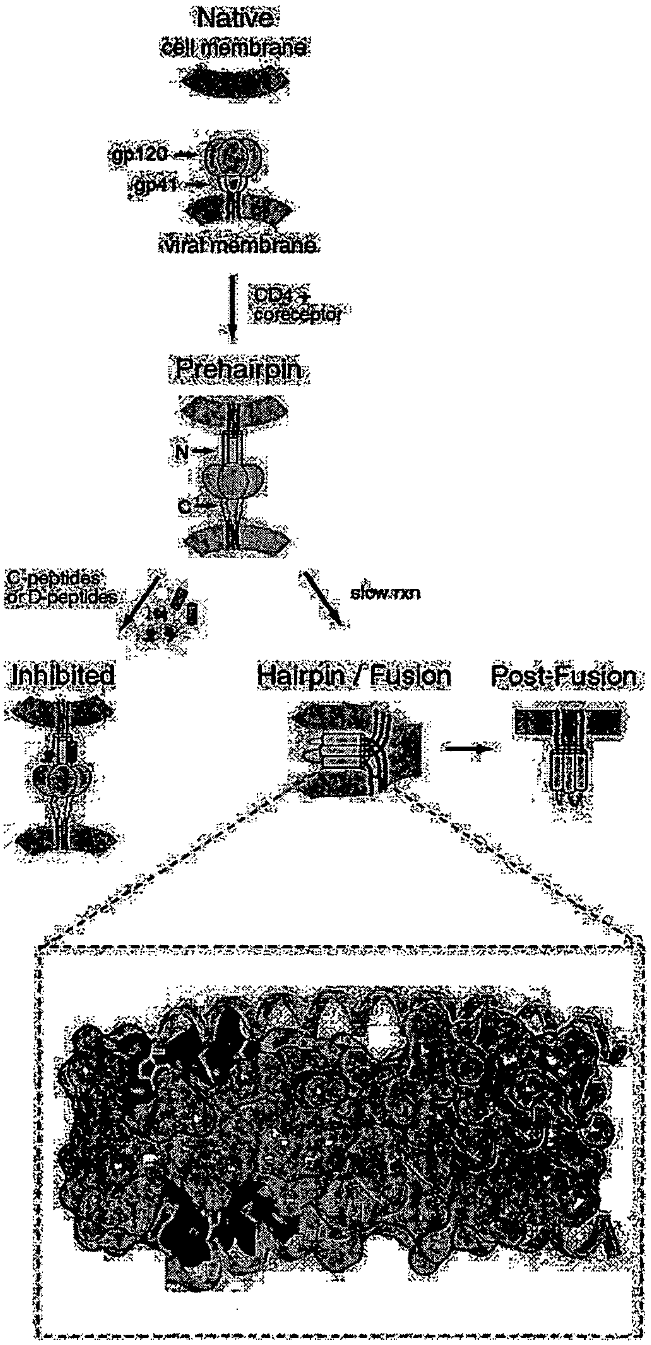
Figure 4 Model of the prehairpin intermediate and inhibitors. After the HIV-1 envelope protein (Env) binds CD4 and the coreceptor, a transient intermediate is formed in which gp41 spans both the viral and the cell membranes. The N-peptide region of gp41 (gray) is exposed and vulnerable to inhibitors. The N-peptide trimeric coiled coil contains three grooves that can be bound by C-peptides (65, 78, 80, 81). It also contains three prominent hydrophobic pockets at its base that can be targeted by potential small-molecule entry inhibitors, such as D-peptides (132). Binding of either C-peptides or D-peptides to the transient intermediate ultimately leads to irreversible inactivation of membrane fusion. Two additional types of inhibitors, 5-Helix (88) and IQN17-like molecules (DM Eckert & PS Kim, unpublished observations), bind the C-peptide region of gp41 (yellow), also inhibiting fusion. Although 5-Helix and IQN17 are depicted binding to the prehairpin intermediate, it is unknown whether these inhibitors target the prehairpin intermediate, the native state of gp41, or both.

formation of the trimer-of-hairpins. However, it is unknown whether the C-peptide is exposed prior to or during the formation of the prehairpin intermediate.

A Common Fusion Mechanism

With the high-resolution structures and other data described above, it is possible to describe a common model for both HA- and Env-mediated membrane fusion. The model described below is derived from several current hypotheses (2, 9, 12, 16) (Figure 5) and considers current experimental data.

In cells infected by either virus, the viral envelope glycoprotein is expressed as an unprocessed precursor, unable to fuse. Subsequently, the precursor is proteolytically processed, locking the protein into a metastable state. The structure of the HA0 unprocessed precursor, recently solved by X-ray crystallography (89), is almost identical to the native, processed form, differing primarily in the 18 residues surrounding the cleavage site. In the precursor, these residues are exposed


Native
cell membrane
gp120
gp41
viral membrane
CD4 + coreceptor
Prehairpin
N
C
C-peptides or D-peptides
slow rxn
Inhibited
Hairpin / Fusion
Post-Fusion

to solvent and folded as an extended, uncleaved loop. On cleavage, the newly created carboxy terminus of HA1 and amino terminus of HA2 separate. The fusion peptide is deeply buried in an interior region at the base of HA, primed for low-pH–activated structural rearrangement.

Data suggest the cleavage event locks the fusion glycoprotein into a metastable state, blocked from its most stable fold by a kinetic barrier. For example, the same HA conformational changes caused by exposure to low pH can also be activated at neutral pH by high temperature or urea denaturation (64). Mutants of HA that mediate fusion at a higher pH than wild type also require a lower temperature than wild type to fuse at neutral pH (90), consistent with metastability of the native state. In addition, when HA2 is expressed in the absence of HA1, it adopts the fusogenic structure (62, 73). Most likely, when HA0 folds as a single-chain precursor, it folds to its energetically most stable state (64). On proteolytic processing, that state becomes the metastable state for the cleaved molecule, essentially priming the virus for fusion.

When the processed protein is exposed to fusion-activating conditions, it overcomes the kinetic barrier of the native state and initiates the conformational changes necessary for fusion. In influenza, the HA1 domains lose their trimeric contacts and slightly dissociate from the compact glycoprotein structure. The fusion peptide is liberated from its buried position and propelled in a spring-loaded fashion to the amino terminus of the central trimeric coiled coil. Now exposed, the fusion peptide can interact with the host-cell membrane. In HIV-1, binding of

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Figure 5 Schematic representation of the current working model for viral membrane fusion. In the native state of the fusion protein, most of the exposed surface area is composed of the surface subunit. Much of the transmembrane subunit, including the fusion peptide, is not exposed. Following fusion-activating conditions (binding of CD4 and coreceptor for HIV-1 and low pH for influenza), conformational changes occur to free the fusion peptide from its unexposed location. For influenza hemagglutinin (HA), this occurs via a "spring-loaded" mechanism. In HIV-1, it is unknown whether fusion peptide release is spring loaded or whether the fusion peptide is simply uncovered by the movement of gp120. At least in HIV-1, the conformational changes result in the formation of the transient prehairpin intermediate. Such an intermediate is also likely to exist in HA-mediated fusion. The prehairpin intermediate spans two membranes, with its transmembrane region in the viral membrane and the fusion peptide deposited in the host-cell membrane. The N-peptide coiled coil, and likely the C-peptide region, is exposed, vulnerable to inhibitory molecules, at least for HIV-1. The prehairpin intermediate of HIV-1 constrains gp41 such that the N- and C-regions cannot interact. In the absence of inhibitors, the prehairpin intermediate resolves into the trimer-of-hairpins, and membrane fusion occurs. (*Inset*) The high-resolution crystal structure of HIV-1 gp41 (67). The N-peptide trimeric coiled-coil core is shown as a surface representation, whereas the C-peptides are depicted as helices (yellow). Three residues from each C-peptide bind to a small hydrophobic pocket (dark gray) that is the target for HIV-1 entry inhibitors (132). Inset drawn with Insight II 98.0 (Molecular Simulations, Inc).

gp120 to CD4 causes conformational changes in gp120, allowing attachment to the coreceptor, followed by further conformational changes in both gp120 and gp41 that greatly weaken their interaction. The transient prehairpin intermediate of gp41 is formed, freeing the previously buried fusion peptide to interact with the host-cell membrane and exposing the N-peptide region (and possibly the C-peptide region). It is unknown whether, as in influenza, there is a spring-loaded mechanism involved in forming the prehairpin intermediate in HIV-1, or whether the fusion peptide maintains the same position as in the prefusogenic state and is simply uncovered by the movement of gp120.

Analogous to HIV-1 Env, it seems likely that influenza HA also progresses through a transient intermediate state—after the spring-loaded mechanism but before the formation of the hairpin. When HA2 is exposed to low pH in the presence of target membranes that contain radioactively labeled, photoactivatable cross-linking reagents, only the fusion peptide becomes labeled (75), and evidence suggests that the labeling of HA2 precedes membrane fusion (91). In the absence of a target membrane, HA quickly becomes inactivated after acidification (76, 77). In the inactive state, the fusion peptide interacts with the viral membrane (75), probably forming the fusogenic structure anchored solely in the viral membrane.

In both viruses, after the fusion peptide of the glycoprotein inserts into the target membrane, the transmembrane subunit spans two membranes, those of the virus and those of the cell. In the fusogenic structure, both termini of the transmembrane subunit ectodomain are located at the same end of the folded molecule. This structure is extremely stable and presumably represents the most thermodynamically favorable form of the molecule. The progression from the transient intermediate to the energetically favorable fusogenic conformation brings the two membrane-proximal regions close together. It is likely that the energy gained by forming this highly stable structure overcomes the unfavorable process of pulling two phospholipid membranes into close proximity. To juxtapose the two membranes and maintain membrane contact on both ends, the fusion glycoprotein likely tilts over and lies parallel to both membranes. Flexibility in the membrane-proximal regions likely assists the tilting of the molecule. Ultimately, both transmembrane regions of the fusion glycoprotein (the fusion peptide and the original transmembrane helix) occupy the same membrane as the viral and cellular membranes become one.

The above description is a simplistic model of a complicated process. The molecular details that drive membrane juxtaposition to membrane fusion are only beginning to be discerned. A small sampling of those details is described here.

Electrophysiological experiments show that fusion between the influenza and host-cell membranes involves a flickering pore that expands over time to allow the release of the viral contents into the cell (92–94). Several pieces of evidence suggest that multiple HA molecules are required to gather at the fusion site to promote pore formation. First, there is a lag time between acidification and pore formation, and this lag time is shown to depend cooperatively on the density of HA on the cell (95). Second, when HA is present at low amounts on the cell, hemifusion (the fusion of the outer monolayers of the membrane in the absence of inner monolayer fusion) occurs (96). Third, quick-freeze electron microscopy reveals an ordered array of HA trimers at the fusion site (97).

It has been proposed that pore formation occurs through a lipid intermediate in which the outer monolayers fuse first, likely forming a stalk between the two enveloped entities (98), followed by formation of the pore when the inner monolayers fuse (99–103). Indeed, the same lipid compositions that promote or inhibit stalk/pore formation likewise affect HA-mediated fusion (99, 101, 103).

OTHER MEMBRANE FUSION PROTEINS

Fusion Proteins of Other Enveloped Viruses

During the past several years, many X-ray crystal structures of enveloped viral fusion protein cores were published (67–70, 72, 104–109) (Figure 6). The four viral families with the most extensive structural information are orthomyxovirus, retrovirus, paramyxovirus, and filovirus. Most of the new structures depict a similar theme—the trimer-of-hairpins described above for influenza and HIV-1. In these structures there is a characteristic central trimeric coiled coil, presumably posed to present the fusion peptide at its tip. At the base of the coiled coil, the chain folds back, and supporting structures bind to the outside of the coiled coil; in most cases, at least part of the outer structure contains a helix. Because the structures for such a diverse array of fusion glycoproteins are so similar, it is likely these viruses utilize a similar mechanism of fusion, as described above.

Computational methods predict that many of the viral fusion glycoproteins not yet studied by high-resolution structural methods also form the trimer-of-hairpins. Heptad repeat regions were identified in many viral envelope proteins over a decade ago (110–113). However, an improved iterative method called LearnCoil-VMF (114), using a database of viral membrane fusion proteins with potential coiled-coil regions, provides a broader, more accurate prediction for many viruses within the retrovirus, paramyxovirus, and filovirus families. LearnCoil-VMF predicts N- and C-helical regions in the fusion glycoproteins of most paramyxoviruses and the lentivirus genus of retroviruses. Therefore, most of these proteins likely form the helical trimer-of-hairpins seen in HIV-1 (67–69) and SIV (70, 71) (Figure *6a*). Indeed, even though paramyxovirus fusion glycoproteins contain more than 200 residues between the predicted helical regions, recent X-ray crystallography studies confirm formation of the trimer-of-hairpins structure (104, 115) (Figure *6b*). In filoviruses and the remaining genera of retroviruses [mammalian C-type, avian C-type, D-type, and bovine leukemia virus—human T-cell leukemia virus (BLV-HTLV)], only the N-helical region is predicted by LearnCoil-VMF. This prediction is confirmed in the X-ray crystal structures of the glycoproteins of Moloney murine leukemia virus (a mammalian C-type retrovirus), human T-cell leukemia virus

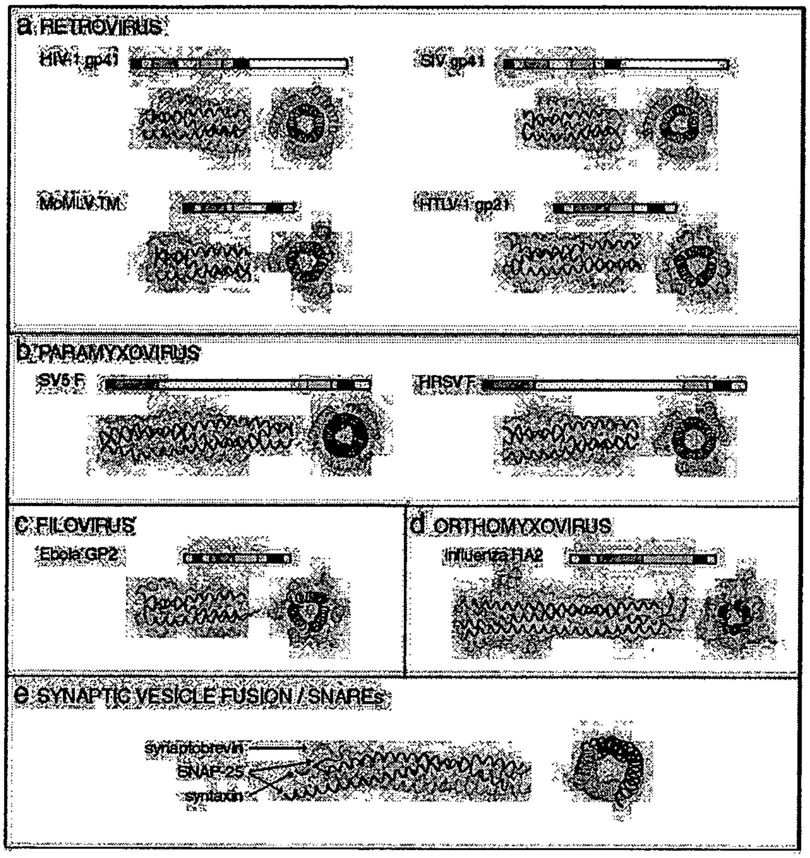

Figure 6 Trimer-of-hairpin structures of the transmembrane subunits of viral fusion glycoproteins and the coiled-coil bundle of the fusion proteins of synaptic vesicle fusion. (*a–d*) The primary and high-resolution structures of eight enveloped virus fusion glycoprotein transmembrane domains shown approximately to scale. In each primary structure, the fusion peptide (red), N-peptide region (blue), C-peptide region (yellow), and transmembrane domain (black) are depicted. In the trimer-of-hairpins side views, the amino- and carboxy-terminal ends of the fusion proteins are located at the same end of the folded structure (right). The top view is looking down from the amino terminus of the N-peptide coiled-coil core. (*a*) The retrovirus family: HIV-1 gp41 (67; see also 68, 69), simian immunodeficiency virus (SIV) gp41 (70; see also 71), Moloney murine leukemia virus transmembrane subunit (TM) (105), and human T-cell leukemia virus type-1 gp21 (106). (*b*) The paramyxovirus family: simian parainfluenza virus 5 F (104) and human respiratory syncytial virus F (115). (*c*) The filovirus family: Ebola gp2 (107; see also 109). (*d*) The orthomyxovirus family: influenza HA2 (72; see also 63). (*e*) High-resolution structure of the SNAREs involved in synaptic vesicle fusion (124) (side view and top view). (Blue) The plasma membrane SNAREs (syntaxin and SNAP-25); (yellow) the vesicle SNARE (synaptobrevin). High-resolution structures drawn with Insight II 98.0 (Molecular Simulations Inc).

(a BLV-HTLV), and the filovirus Ebola, in which the only substantial stretches of helix are composed of the N-peptides (Figure 6*a,c*). Nonetheless, these structures still contain the trimer-of-hairpins. The N-peptide region forms the familiar trimeric coiled-coil core, but the surrounding C-peptides pack around the N-peptide core in a more extended conformation with only short helical stretches, similar to HA (105–107, 109) (Figure 6*d*).

Enveloped viruses are known to cause many serious human diseases and disorders. For example, human respiratory syncytial virus, a paramyxovirus, is a major cause of bronchiolitis and pneumonia in infants and young children. Ebola, a filovirus, can cause a severe form of hemorrhagic fever. Because these and many other viruses likely have a similar fusion mechanism to that described in this review, they may be vulnerable to similar mechanisms of inhibition. It is interesting that the C-peptide regions of many of the paramyxoviruses, such as Sendai virus, measles, Newcastle disease virus, human parainfluenza virus, respiratory syncytial virus, and simian parainfluenza virus 5, can be utilized to inhibit virus infectivity (116–122). Additional methods for inhibiting HIV-1 entry, described below, may also apply to other enveloped viruses.

Vesicle Fusion Proteins

The coiled-coil helical bundle may be a global motif for promoting membrane fusion events. Recent evidence suggests that vesicle fusion is also mediated by formation of a coiled-coil structure that juxtaposes the fusing membranes (123, 124; for a review, see 125).

Vesicles move macromolecules from one membrane-bound cellular compartment to another through a series of membrane budding and fusion events. In exocytosis, vesicles fuse with the plasma membrane from within the cell to release macromolecules. For example, in synaptic vesicle exocytosis, the vesicle fuses with the plasma membrane of a neuron to release neurotransmitters into the synapse. A group of proteins called SNAREs, located on both the vesicle and target membranes, mediate fusion. In synaptic vesicle exocytosis, the vesicle contributes one SNARE (synaptobrevin), and the plasma membrane contributes two (syntaxin and SNAP-25).

X-ray crystallography studies suggest that the SNAREs of synaptic vesicle fusion mediate membrane fusion through the formation of a coiled-coil structure. Three SNARES form a highly stable bundle of four helices (124) (Figure 6*e*). (Each SNARE contributes one helix, except SNAP-25, which provides two.) The membrane-proximal regions are located at the same end of the helical bundle, just as in the trimer-of-hairpins of the viral protein. It is proposed that this bundle enables juxtaposition of the two membranes, leading to membrane fusion (123, 124).

Although vesicle exocytosis and viral membrane fusion are distinct biological processes that utilize diverse proteins for fusion, the underlying mechanism appears similar—the use of a coiled-coil structure to juxtapose the membranes. Quite possibly, coiled-coil domains that participate in additional membrane fusion events will be identified.

INHIBITING HIV-1 ENTRY

Since the discovery of HIV two decades ago, over 20 million deaths have been attributed to acquired immune deficiency syndrome (AIDS). Currently, over 36 million people worldwide are infected with the virus, corresponding to one HIV-positive person for every 200 people in the world (25a). At the end of 2000, the 16 anti-HIV-1 drugs approved by the Food and Drug Administration target only two viral proteins. The two current viral targets are reverse transcriptase, which is responsible for transcribing the HIV-1 RNA genome to DNA, and protease, which processes the HIV-1 Gag/Pol polyprotein and the subsequent Gag protein (126). Because of the high rate of viral turnover (127) and the error-prone nature of reverse transcriptase, viruses resistant to these small-molecule drugs often emerge. Currently in the United States, combination therapy, in which three or more drugs are administered concomitantly, is the routine treatment. Although combination therapy is often successful at lowering viral load, there are significant problems associated with it. Some patients develop immediate adverse effects and are therefore intolerant of available drugs. Patients who are more tolerant face expensive, arduous treatment. Among these patients, some harbor viral strains resistant to several drugs (128), and long-term adverse effects of treatment can develop (126, 129, 130). Also, because of increasing viral resistance, the threat of an outbreak of a virus immune to all available drugs is rising (128). Therefore, drugs that target an additional step of the viral life cycle, such as viral entry, would be useful, especially if they are less toxic and less susceptible to viral resistance than current therapies.

The increased understanding of viral entry opens the door to the design and discovery of HIV-1 entry inhibitors (16, 22, 23, 25). As there are multiple protein molecules involved in the entry process, both on the virus and on the host cell, there are multiple potential targets for intervention. As mentioned, the inhibitory activity of the C-peptides demonstrates the feasibility of targeting the transient gp41 structure that emerges during viral infection. Indeed, several additional inhibitors were discovered that target gp41 prior to the formation of the trimer-of-hairpins (88, 131–133) (see below). Also, the recent identification of the HIV-1 coreceptors (for a review, see 18), as well as the completion of a high-resolution structure of gp120 core bound to CD4 and an antibody mimicking the coreceptor (134) (see below), made it possible to discover molecules that inhibit HIV-1 from binding to the cell.

Anti–HIV-1 molecules that inhibit entry stop the virus before it infects the host cell, unlike currently used drugs that act only after infection has occurred. Identified drugs that stop the virus from invading cells may be useful as prophylactic agents, creating a barrier to the initial infection event. The knowledge utilized for the design of drugs to inhibit HIV-1 entry could also provide useful leads for effective HIV-1 vaccines.

Targeting the Transient Fusion Intermediate

The gp41 transient intermediate of viral entry is a promising target for inhibition (16). C-peptides, which bind to this intermediate, have shown reasonable success in human clinical trials as injected therapeutics (135). Participants who received 100 mg of the C-peptide T20 twice daily experienced viral reduction levels similar to patients treated with one reverse transcriptase or protease inhibitor.

There are disadvantages, however, to the therapeutic use of C-peptides. First, because of their size, C-peptides are not amenable to oral routes of entry and must be injected. Second, producing the lengthy C-peptide requires expensive chemical synthesis, and large amounts of the peptide are required to observe an antiviral effect in humans. It would be preferable to identify an orally bioavailable small molecule that mimics the function of the C-peptide as an alternative therapy.

Recent progress has been made toward identifying such molecules (for a review, see 24). In the gp41 X-ray crystal structure there is a small pocket in the conserved hydrophobic groove of the N-peptide trimeric coiled coil (Figure 5). Three hydrophobic residues from the C-peptide, two tryptophans and an isoleucine, bind this pocket. The pocket was proposed to be an attractive target for drug discovery for many reasons (67, 83). First, the pocket is small (400 Å^3) and provides a structurally defined binding surface, ideal for binding by a small molecule of 500–600 Da. Second, many of the residues lining the pocket are critical for membrane fusion (38, 40, 42–45). Third, C-peptide inhibitory activity depends on its ability to bind the pocket (83). Fourth, drugs that target this pocket may elude the emergence of resistant virus because (*a*) the residues that constitute the pocket are highly conserved among all known HIV-1 isolates as well as among SIV isolates, and (*b*) the mRNA encoding this region is an integral part of the structured Rev-response element (136, 137), which suggests there is selective pressure not to mutate at both the protein and RNA levels. Finally, although there are effective C-peptides that do not contain pocket-binding residues (e.g. T20), such C-peptides are more vulnerable to the emergence of resistant viruses than are those containing the pocket-binding residues (e.g. T649) (85).

Three methods to identify pocket binders have been reported (131–133). In one attempt, utilizing the X-ray crystal structure of the gp41 core and molecular docking techniques, a database of 20,000 small organic molecules was screened for potential fitting into the hydrophobic pocket. Of the 16 compounds with the best fit, two were reported to inhibit formation of the trimer-of-hairpins, with one inhibiting HIV-1 infection at micromolar concentrations (131). Further work is required to show that this compound is indeed binding to the pocket region of the N-peptide. A second method used a combinatorial chemistry approach. C-peptides with a combinatorial library of three organic chemical moieties replacing the pocket-binding region were screened for improved binding to N-peptides

(133). One member of the library improved the inhibitory activity of a 30-residue C-peptide but was not able to inhibit HIV-1 infection on its own. A third method utilized mirror-image phage display (138) to identify small peptides composed of D-amino acids that bind to the pocket (132).

The target for the mirror-image phage display was designed to overcome a potential hurdle in identifying pocket binders. gp41 N-peptides aggregate in the absence of C-peptides (65, 139) and therefore are not good targets for identifying pocket-binding ligands. A hybrid coiled-coil molecule, IQN17, was designed to avoid aggregation and properly present the gp41 pocket. In IQN17, a soluble trimeric coiled coil derived from GCN4-pI$_Q$I (140) is fused to the pocket-forming residues of gp41. IQN17 is helical, soluble, and trimeric, as shown by biophysical studies. Peptides were identified that bind to IQN17 but that do not bind to a control molecule with a point mutation that occludes the pocket. A 1.5 Å cocrystal structure and a simple nuclear magnetic resonance assay demonstrate that the D-peptides bind specifically to the gp41 pocket region of IQN17. These D-peptides inhibit HIV-1 infectivity at micromolar concentrations. Because of low potency, the D-peptides themselves may not prove useful for therapy, although their identification has validated the concept that targeting the gp41 coiled-coil pocket, and only the pocket, is a viable therapeutic option. In addition, the D-peptides in combination with the IQN17 target that accurately represents the gp41 pocket provide key tools for identifying other potentially useful pocket-binding compounds.

Targeting the C-Peptide Region of gp41

As mentioned, N-peptides derived from gp41 exhibit anti–HIV-1 characteristics at micromolar concentrations (65, 79), but the mechanism of N-peptide inhibition is not known. N-peptides have a strong tendency to aggregate (65, 139) and may either target the C-peptide region of gp41 (65) or intercalate into the gp41 amino-terminal coiled coil (44, 79).

To determine whether the C-peptide region is a useful target for inhibiting the formation of the trimer-of-hairpins structure, a new inhibitor, denoted 5-Helix, was recently designed (88). 5-Helix is a 25-kDa protein consisting of five of the six helices of the gp41 trimer-of-hairpins joined by short peptide linkers. The design harnesses the C-peptide binding ability of N-peptides while reducing their tendency to aggregate. 5-Helix, which lacks a third C-peptide, binds to C-peptide with high affinity and inhibits a variety of HIV-1 strains at nanomolar concentrations. A control molecule, 6-Helix, consists of the entire trimer-of-hairpins structure and does not bind C-peptide or inhibit HIV-1 infection. With its ability to specifically target the C-peptide region of gp41, 5-Helix demonstrates a new avenue for inhibiting HIV-1 entry (Figure 4).

In a similar approach, hybrid coiled-coil peptides that decrease the aggregation of N-peptides dramatically increase the anti–HIV-1 inhibitory activity of the N-peptides (DM Eckert & PS Kim, unpublished data). A panel of hybrid coiled-coil peptides was synthesized in which one of two stable, soluble, trimeric coiled coils (GCN4-pI_QI or IZ) was fused to the amino terminus of N-peptides of varying lengths. For example, IZN17 is composed of a designed trimeric isoleucine zipper (IZ) fused to 17 residues of the N-peptide. The hybrid coiled coils increase the inhibitory activity of the comparable N-peptide by two to three orders of magnitude, with the best inhibitor working at low nanomolar concentrations. Like 5-Helix, these peptides likely inhibit by binding to the C-peptide region of gp41 (Figure 4).

High-Resolution View of gp120

Recently, the high-resolution structure of gp120 bound to CD4 and a molecule mimicking the coreceptor was described (134; for review, see 141, 142) (Figure 7). The structure provides atomic detail on potential binding sites for entry inhibitors as well as suggestions as to why targeting these sites may prove difficult.

The primary sequence of gp120 is composed of five regions of high sequence variability among known HIV-1 isolates (termed V1–V5) interspersed with five conserved regions. The X-ray crystal structure shows a mostly deglycosylated gp120 conserved core (missing V1, V2, V3, and the amino and carboxy termini) bound to two domains of CD4 and to the antigen-binding fragment (Fab) of the monoclonal antibody 17b that binds to the coreceptor recognition site. gp120 contains two structural domains, an inner domain, presumed to contain the gp41 contacts, and an outer domain. These regions are joined by a small "bridging" domain (Figure 7).

The CD4 binding site is large and located at the interface of all three gp120 domains. The binding surface contains both conserved residues required for CD4 binding as well as variable residues. CD4 interacts with the variable residues through hydrogen bonding to main-chain atoms. Also, cavities exist within the binding surface that are not contacted by CD4. Immune recognition of this CD4 binding site is probably difficult for three reasons: (*a*) There is a mix of conserved and variable regions in the binding site, (*b*) the V1 and V2 regions likely mask the CD4 binding site prior to binding (143), and (*c*) the CD4-bound state may represent an otherwise energetically unfavorable conformation of gp120 (134, 142). For these same reasons, it may also be difficult to target this area with therapeutic molecules.

The Fab fragment binds to the side of the bridging domain composed of part of the fourth conserved domain and the base of the V1 and V2 stem. This region is presumed to be the coreceptor-binding site. Although the V3 loop is absent in the X-ray crystal structure, it seems likely that this region is shielded by the V3 loop before CD4 binding. First, in the high-resolution structure, there is a large gap between the core gp120 and the 17b antibody light chains that would most likely be filled by the V3 loop (143). Second, antibodies that recognize the V3 loop can inhibit coreceptor binding (55). Third, V3-recognizing antibodies compete for gp120 binding with the neutralizing antibodies, such as 17b, that recognize the coreceptor binding site (144).

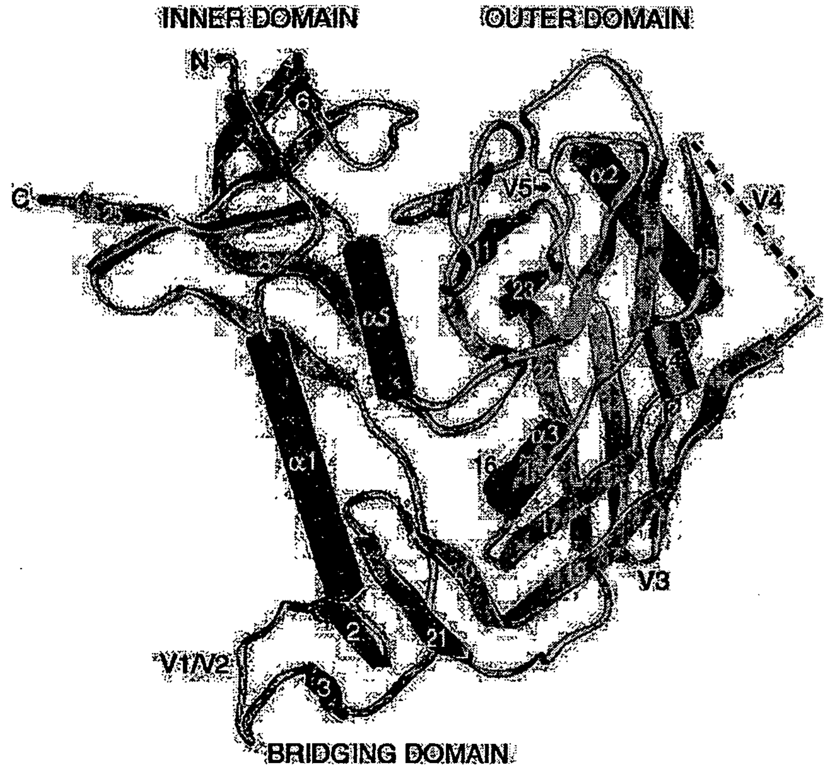

Figure 7 High-resolution X-ray crystal structure of core gp120. In this view, the CD4 binding site is facing the viewer, and the coreceptor-binding site is at the bottom of the structure. (Pink) α-Helices; (purple) β-sheets. The inner domain, which presumably contacts gp41, is composed of β-sheets 1, 4–8, and 25 as well as α-helices 1 and 5. The outer domain is composed of β-sheets 9–19 and 22–24 and α-helices 2–4. The bridging domain is composed of α-helices 2, 3, 20, and 21. The V1/V2 and V3 variable regions were both replaced with Gly-Ala-Gly tripeptide sequences. The positions of the V1/V2 and V3 substitutions are indicated. V4 was disordered in the structure (dashed line). The amino and carboxy termini of core gp120 are indicated. Figure drawn with Insight II 98.0 (Molecular Simulations, Inc). (Adapted from Reference 134.)

Inhibiting the gp120/CD4 Interaction

Early efforts to inhibit entry of HIV-1 focused on inhibiting binding of the envelope protein to CD4 on the surface of cells by competition with a soluble version of CD4. Soluble CD4 (sCD4) is composed of the ectodomain of CD4 and inhibits laboratory-adapted strains of HIV-1 (58, 145). When used against primary isolates, however, sCD4 was much less successful and actually increased the infectivity of some isolates (for a review, see 146). In laboratory-adapted strains, sCD4 induces shedding of gp120 from gp41 (37), thereby ablating the virus's potential to bind to and fuse with host cells. The same extent of shedding is not observed in primary isolates that have a lower affinity for sCD4 (147). In addition, the initial interaction of gp120 with CD4 induces changes in gp120 that allow it to bind the coreceptor with higher affinity (59, 148, 149; for a review, see 18). Indeed, as mentioned above, after exposure to sCD4, HIV-1 Env can mediate fusion with cells expressing the coreceptor but lacking CD4 (59). Thus, in primary isolates, when gp120 shedding does not occur, sCD4 likely promotes fusion by facilitating binding to the coreceptor.

Recent designs of sCD4-like molecules, however, demonstrate promising results. Pro542 is a tetrameric version of sCD4 in which the gp120-binding region of CD4 is fused to the constant region of the human immunoglobulin, IgG2 (150). It has an increased affinity for gp120 and has shown some success in decreasing viral loads of HIV-1–infected patients in phase I clinical trials (151). Lower-molecular-weight, sCD4-like molecules were made by grafting critical gp120-binding motifs from CD4 onto toxins (peptides of ~30 residues) of similar structure (152, 153). The toxin chimeras are capable of mimicking CD4-induced conformational changes in gp120 (152) and inhibiting CD4 binding and viral infectivity (153).

There are several other molecules undergoing preclinical or clinical evaluation that interfere with the gp120/CD4 interaction (for reviews, see 22, 23, 25). FP213999, Zintevir, and cyanovirin-N are anti–HIV-1 molecules identified for their ability to inhibit viral infection in vitro (154–156). Subsequently, each was proposed to inhibit the gp120/CD4 interaction (157–159). PRO 2000, a 5-kDa napthalene sulfonate polymer, was shown to inhibit gp120/CD4 interaction in an ELISA assay (160). Despite their ability to inhibit the gp120/CD4 interaction in vitro, the antiviral activities of cyanovirin-N and PRO 2000 are nonspecific (157, 161). Ultimately, any of these molecules that do not demonstrate ideal behavior as therapeutics in clinical studies may be useful as topical preventive medications.

Chemokine Receptors

Chemokine receptors provide an additional potential therapeutic target. Most HIV-1 variants use one or both of two specific chemokine receptors, CXCR4 and CCR5 (for a review, see 18, 19, 162), for cell entry. CXCR4 is expressed on T-cells and is therefore utilized by T-cell–tropic HIV-1 (now called X4 isolates). CCR5 is expressed on macrophages and is consequently the coreceptor of choice for macrophage-tropic HIV-1 (or R5 isolates). Both receptors are expressed on primary T-cells. These cells are vulnerable to infection by R5, X4, and dual-tropic (R5X4) viruses. Although CXCR4 and CCR5 are the two most common coreceptors, at least 11 additional coreceptors have been reported. It is not known whether any of the additional coreceptors have significance in vivo.

The chemokine receptors are attractive targets for several reasons. First, the chemokine receptors are static targets not prone to mutation, in contrast to viral targets. Second, rare individuals with homozygous deletions in the CCR5 gene are resistant to R5 virus infection (163, 164). Third, each coreceptor has specific chemokine ligands, and these ligands are effective in blocking entry of HIV-1 isolates that utilize that specific coreceptor. For example, the chemokine ligands of CCR5 (RANTES, MIP-1α, and MIP-1β) are effective inhibitors of R5 HIV-1 strains (165–168), whereas SDF-1α inhibits CXCR4-mediated entry (169, 170).

Treatment with chemokines themselves may cause unfavorable side effects. Chemokines bind chemokine receptors to guide the movement of white blood cells during an immune response to injury or infection. Therefore, the use of chemokines as therapeutic molecules may cause unwanted activation or interference with normal signaling of the inflammatory pathway. In response to this concern, chemokine derivatives that uncouple viral inhibition activity from natural receptor function were created (171–175). For example, a RANTES derivative lacking eight amino-terminal amino acids no longer activates chemotaxis, yet is only slightly less effective at inhibiting CCR5-mediated HIV-1 entry (171). Also, in a step toward potent, small-molecule drugs, several low-molecular-weight compounds were identified that bind to either CXCR4 or CCR5 and inhibit HIV-1 infection (176–178). A bicyclam, AMD3100, is the most advanced in therapeutic trials (178).

It is unknown whether the targeting of chemokine receptors will be a successful therapeutic approach. Although individuals with homozygous deletions in CCR5 do not exhibit any negative side effects (163, 164), CXCR4 knockout mice exhibit an embryonic-lethal phenotype (179, 180). In addition, it is possible that blocking specific coreceptors will lead to a possibly detrimental selection for viruses with altering tropism. In a SCID-hu mouse model sensitive to HIV-1 infection, a rapid switch in coreceptor usage was seen after a short treatment with an anti-CCR5 agent (174), and in an HIV-1 infection, a switch to an X4 viral population usually coincides with the onset of AIDS.

HIV-1 Vaccine Strategies Targeting gp120 and/or gp41

A safe vaccine that could be widely administered and could either prevent or decrease the rate of infection would be the most useful method for combating new infections in the world. Current vaccine efforts are twofold: (*a*) to elicit cell-mediated responses that target HIV-1 infected cells, and (*b*) to raise a neutralizing antibody response that effectively targets viruses. Significant progress has been made in generating a cell-mediated HIV-1–specific immune response (181, 182). Rodents and nonhuman primates generate HIV-1–specific cytotoxic T-lymphocytes in response to a variety of immunogens, including live recombinant vectors, HIV-1 peptides, and plasmid DNA–encoding HIV-1 proteins. Raising an effective neutralizing antibody response has been much more elusive.

Only three antibodies that potently neutralize a wide range of HIV-1 isolates in vitro have been isolated from HIV-1–infected patients—two specific for gp120 epitopes (b12 and 2G12) and one that recognizes a gp41 epitope (2F5) (183–187).

Passive transfer of a mixture of these antibodies can successfully protect Rhesus macaques against challenge by a SHIV containing either a laboratory-adapted or a primary isolate HIV-1 Env (188, 189). However, neutralizing antibodies were administered at extremely high levels (ranging from 30–400 mg/kg) to observe this effect.

Although the HIV-1 Env protein is extremely immunogenic, attempts to raise potent neutralizing antibodies in the laboratory against a broad range of HIV-1 viruses with viral and protein immunogens have been largely unsuccessful (for reviews, see 190–192). This is likely due to a variety of reasons. First, much of the sequence of Env is highly variable between viral strains, and therefore neutralizing antibodies are often strain-specific. Second, the high mutation rate of the virus likely allows quick escape from potentially neutralizing antibodies. Finally, most protein immunogens used thus far have probably not properly represented the trimeric conformation of Env found on the surface of the virus.

Recent efforts attempt to reproduce the native structure of Env. For example, Binley et al (193) created a stable gp120/gp41 complex, SOS gp140, with an engineered disulfide bond to keep the two subunits covalently associated. The antigenic profile of SOS gp41 is similar to that of native Env. The same disulfide-bonded construct, with deleted variable loops on gp120, may expose hidden conserved epitopes on gp120 (194). In addition, Yang et al (194a) have created soluble stabilized Env trimers that elicit neutralizing antibodies more efficiently.

In a different approach, it was suggested that eliciting an antibody response against transiently exposed conserved conformations of proteins involved in HIV-1 may be successful at neutralizing a broad range of viral strains (132, 195). The gp41 prehairpin intermediate, or other Env conformations, such as the coreceptor-binding site, exposed transiently during the fusion process, may provide useful targets. These regions are likely too transient for HIV-1–infected patients to develop an immune response to them. However, potential immunogens could present these structures in stable, exposed conformations. For example, a covalent gp120/CD4 protein antigen could stably expose the conserved coreceptor-binding site (196). Also, gp120 molecules lacking glycosylation or some of the variable regions may increase the exposure of neutralizing epitopes (197). Additionally, molecules such as 5-Helix (88) and IQN17 (132), which accurately represent the potential transient conformations of gp41, may be able to raise a neutralizing response.

CONCLUSION

Extensive biochemical and structural studies on viruses from different families provide a general mechanism for viral envelope glycoprotein-mediated membrane fusion. Viruses synthesize their fusion glycoproteins in an inactive form. In this state, the fusion glycoprotein adopts a thermodynamically stable conformation.

Subsequently, the fusion glycoprotein is proteolytically processed into two subunits, a surface subunit and a transmembrane subunit. No longer free to sample all conformational space, the processed protein is trapped in the same conformation as the precursor, primed for fusion. The protein waits in a metastable state for the appropriate activation signal, whether an induction of low pH or receptor binding. After the signal arrives, the glycoprotein unleashes its fusion potential. No additional energy, such as ATP hydrolysis, is required. Through a spring-loaded mechanism, at least in the case of influenza HA, the fusion peptide is propelled out of the interior of the protein and inserted into the target membrane. The protein now spans both membranes, and in HIV-1 Env, this prehairpin intermediate is vulnerable to inhibition for many minutes. Subsequently, the protein then adopts its most stable fold, the trimer-of-hairpins. The energy harnessed through acquisition of the stable state likely promotes fusion of the two membranes. Recent evidence on SNARE-mediated synaptic vesicle fusion suggests that coiled-coil helical bundles may be a global motif for promoting membrane fusion events.

The dissection of the viral membrane fusion process has led to a new strategy in HIV-1 therapy development—targeting viral entry. Current efforts attempt to inhibit HIV-1 binding to CD4 and the coreceptor, as well as gp41-mediated membrane fusion. Because many enveloped viruses likely use the same mechanism of entry, similar therapeutic strategies may be effective against a wide range of viral diseases.

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EXHIBIT 5
Appl. No. 10/685,801

O I P E IAP47
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SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION GUIDELINES

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Example 18: Process claim where the novelty is in the method steps.

Specification: The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of β-galactosidase using the claimed method using a cytochrome oxidase promoter.

Claim:

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

Analysis:

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of β-galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Conclusion:

The claimed invention is adequately described.

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